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CEREAL CHEMISTRY

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CONTENTS

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Cereal Chemistry publishes scientific papers dealing with raw materials, processes, or products of the cereal industries, or with analytical procedures, technological tests, or fundamental research, related thereto. Papers must be based on original investigations, not previously described elsewhere, which make a definite contribution to existing knowledge.

Cereal Chemistry gives preference to suitable papers presented at the Annual Meeting of the American Association of Cereal Chemists, or submitted directly by members of the Association. When space permits, papers are accepted from other scientists throughout the world.

The papers must be written in English and must be clear, concise, and styled for *Cereal Chemistry*.

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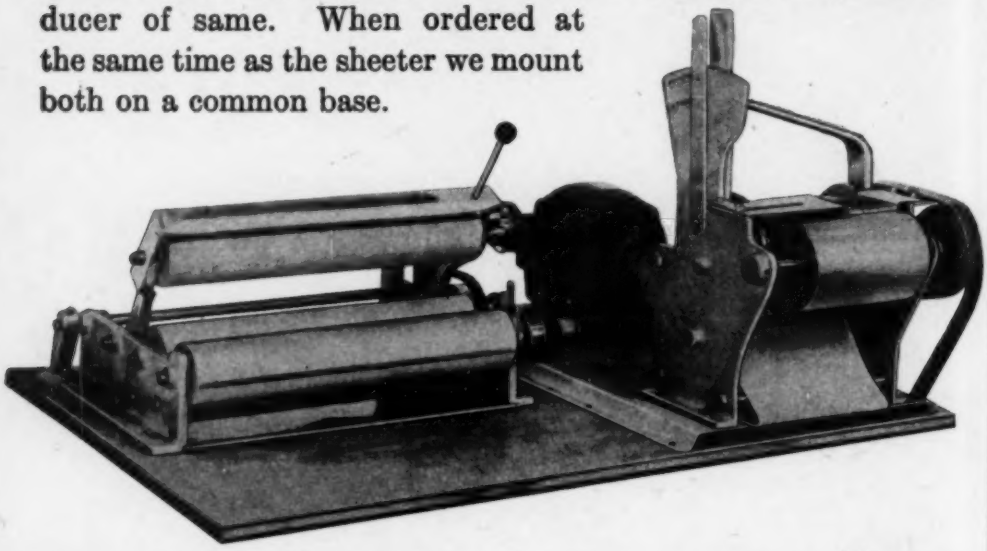

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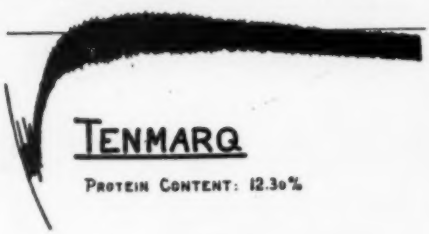

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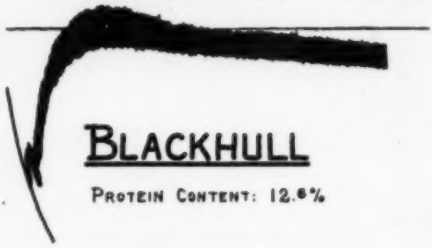

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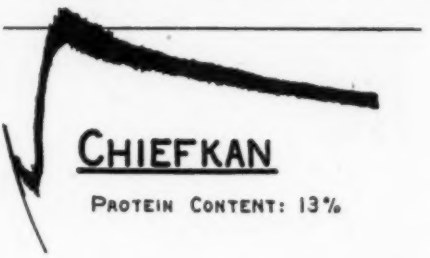

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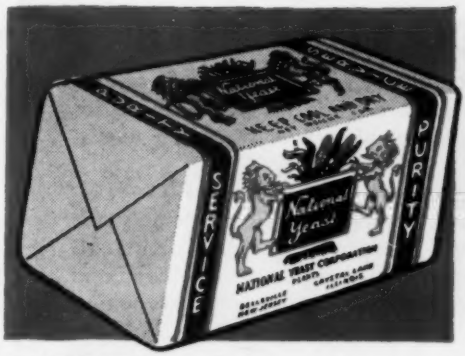

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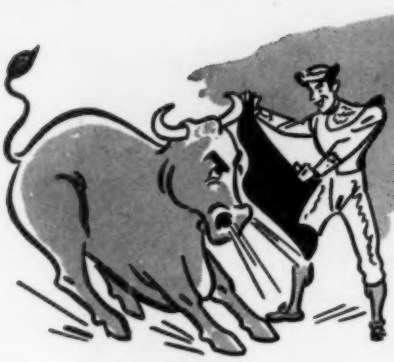

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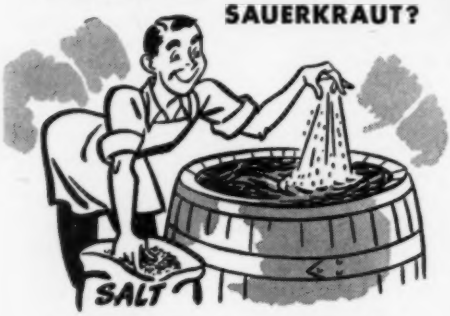

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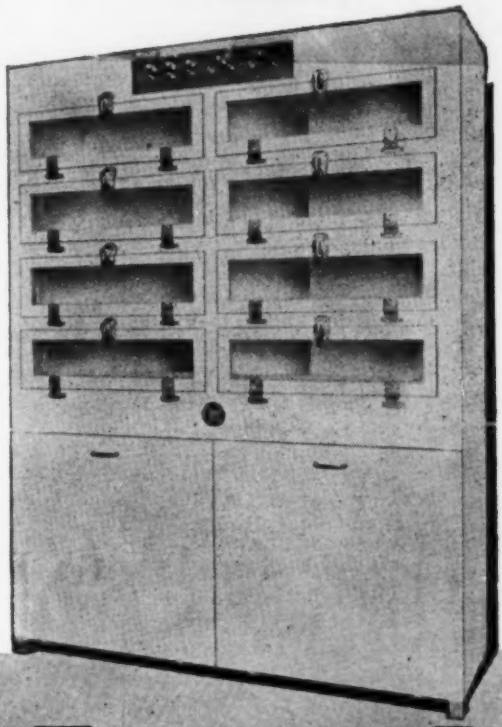

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CEREAL CHEMISTRY

VOL. XXV JANUARY, 1948 No. 1

PREPARATION OF AMYLASE-ACTIVE CONCENTRATES FROM MOLD BRAN[1]

ROBERT L. GATES[2] and ERIC KNEEN[3]

ABSTRACT

In the investigation of the precipitation potentialities of methanol, ethanol, isopropanol, and acetone, it was ascertained that under the proper conditions any of these compounds may be used for amylase precipitation. Methanol requires low temperature (0°C.) operations to obtain satisfactory recovery, while this is not mandatory for the other three compounds. Arranged in order of precipitation efficiency, based on the concentration required to give 90% recovery of amylase activity, they fall into the order: acetone, isopropanol, ethanol, methanol. Methanol and ethanol cause loss of activity when allowed to remain in contact with the enzyme precipitate either in the precipitation operation or in drying. Isopropanol and acetone do not show this property. Separation may be aided by the presence of certain cations. With all precipitants optimum hydrogen ion concentration for precipitation is pH 5.5–6.5. The presence of an excess of multi-valent cations in the extract is detrimental to the activity of the precipitate. Isopropanol is the most tolerant to the presence of these ions.

The ability of certain organic substances to precipitate the enzymes of mold bran from a water extract is well established, but the influence of certain environmental factors upon the enzyme activity and upon the physical characteristics of such precipitates is not so well known. Additional information on these factors not only should help in the commercial processing of mold bran but also should provide valuable information relative to the fundamental properties of the mold amylases.

Many approaches have been made to the problem of purification of the amylases. The majority of the work done in this field has been to obtain a relatively pure concentrate for further studies of the properties of the enzyme.

[1] This paper represents a portion of a thesis presented to the Graduate School of Kansas State College in partial fulfillment of the requirements for the degree of Master of Science. The work was supported by a grant from the Farm Crops Processing Corporation. Contribution No. 143, Department of Milling Industry. Manuscript received October 8, 1947.

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One of the first references to a purification method for amylases is the report of Payen and Persoz (11) that extracts of malt were precipitated with ethanol to concentrate the active principle. A great deal of work has since been done on the use of ethanol as a precipitating agent. Newton and Naylor (10) used 65% ethanol to precipitate the amylase from soybean extracts. The work of Sherman and Schlesinger (14) shows the feasibility of ethanol as a precipitating agent for the concentration of pancreatic amylases. The differential solubility in ethanol of the alpha and beta amylases of malt has been used by numerous workers to obtain relatively pure alpha and beta amylase. The background of this application has been discussed by Kneen, Sandstedt, and Hollenbeck (8). Ethanol has likewise been employed for the purification of mold amylases (17, 3, 9).

The importance of acetone as a precipitating agent can be inferred from its incorporation in the purification methods of Sherman and Schlesinger (15) and Tilden, Adams, and Hudson (19).

The work of Hayasi (4) suggests the use of methanol as a precipitating agent for mold amylases.

As an aid to precipitation of mold amylases the addition of barium chloride to the enzyme infusion prior to precipitation with ethanol is suggested by Takatomi and Takeda (18).

A detrimental effect of ethanol on the activity of the precipitate was reported by Sherman and Schlesinger (16), and Blish, Sandstedt, and Mecham (2).

The present study was prompted by the paucity of information available on the details for the separation of amylase from mold brans and the precipitation of these enzymes in an active, water-soluble, and concentrated form. Such information is a prerequisite to commercial application of the materials as well as to a better understanding of the fundamental nature of the enzymes.

Materials and Methods

In the investigation of precipitation procedures, four organic compounds have been compared under various conditions as precipitating agents, i.e., methanol, ethanol, isopropanol, and acetone.

A quantity of commercial mold bran was obtained from the Mold Bran Company of Eagle Grove, Iowa, and this was used throughout the experiments as the crude product from which the enzyme was extracted for precipitation. This bran was produced by the growth of a strain of *Aspergillus oryzae* on wheat bran. For comparison of enzyme systems, other commercial mold brans were obtained from Wallerstein Company, Jeffreys Laboratories, and Jacques Wolfe Company. Fungal concentrates from Rohm and Haas (RHozyme S),

Wallerstein Company, and Schwarz Laboratories (Polidase S) also were used in some of the comparisons.

The principal amylase in fungal enzyme systems being of the alpha type as pointed out by Kneen and Sandstedt (7), a modified Wohlgemuth (20) method was used to follow precipitation recoveries. This method is based on the time required to obtain the red-brown color with iodine described by Sandstedt, Kneen, and Blish (13). A known amount of extract was allowed to digest a 20-ml. aliquot of 1% boiled soluble starch buffered with sodium citrate-hydrochloric acid buffer at a pH value of 5.0. The time (dT) in minutes required by an appropriate aliquot to convert the substrate at 30°C. to a point where the "red-brown" color was given with iodine was determined.

The aliquots of the extracts or solutions used for activity determinations varied from 2 to 10 ml. depending on activity. Since the total reaction volume was constant, 30 ml., it was necessary to adjust the volume with 0.2% calcium chloride solution (Hollenbeck and Blish, 5), when less than 10 ml. enzyme aliquot were employed. The accuracy of this procedure is about 5%, so only differences in results of this order or greater are considered significant.

The method outlined by Kneen and Beckord (6) was used for comparison of the saccharification action of the various enzyme systems. This method measures the comparative amount of fermentable sugars produced. A starch substrate was digested by the enzyme, the sugars produced fermented by yeast, and this fermentation followed manometrically in the "pressure meter" of Sandstedt and Blish (12).

In order that a greater number of precipitation factors might be studied it was deemed desirable to use a simple and rapid method of following precipitation recovery. Ten ml. of a one to ten extract (one part mold bran to ten parts extractant) of the mold bran were placed in a 100-ml. centrifuge tube and adjusted to the desired set of conditions for precipitation. To this was added a calculated amount of precipitating agent. The precipitate formed was centrifuged out and the supernatant liquid discarded. To the residue were added 40 ml. of distilled water. The dextrinizing activity of an aliquot of this solution was compared with a similar quantity of the original extract, and from this was calculated the per cent recovery. In all subsequent references to recovery of amylase it should be kept in mind that the recovery of amylase was measured in terms of the dextrinizing activity of the redissolved precipitate.

Experimental

After standardizing a method of extraction, the problem of preparing an enzyme concentrate became a process of determining, step

by step, the effect of precipitant concentration, temperature, hydrogen-ion concentration, and the kind and concentration of salt present during precipitation on the activity and physical characteristics of the precipitates.

Extraction of Amylase. The studies on the technique of extraction showed that the total amount of enzyme extracted is constant regardless of the ratio of extraction, but the amount of liquid extract recovered varies inversely with the bran-water ratio in extraction. It was concluded that an extraction ratio of one to ten was most satisfactory for the research. Dilute calcium chloride solutions have been suggested as appropriate extractants for the alpha type of amylase (5). With the particular mold bran used the activity of the extract seemed to be independent of the presence of calcium chloride in the extraction medium. Unless otherwise indicated, all mold bran extractions were made with distilled water.

Three methods of extraction were investigated. Method one was the standard method of Sandstedt, Kneen, and Blish (13). The extraction mixture was allowed to stand for an hour at 30°C. with agitation at 15-minute intervals. Method two made use of a mechanical laboratory stirrer, the extraction mixture being in a beaker in a 30°C. bath. Method three employed a Ward's Liqui-Mixer. A greater concentration of enzyme was obtained from a 3-minute extraction with the Liqui-Mixer than with a 1-hour extraction by the standard method. Fifteen-minute extraction with method two was equal to a 1-hour standard extraction.

A temperature of 30°C. was the most convenient temperature to use, and since satisfactory enzyme infusions were obtained at this temperature, it was used as the standard extraction temperature throughout the work.

The procedure used for removing solid material from the extract was to strain the mixture through coarse cloth and then to centrifuge for the removal of the remaining small particles.

Precipitation of the Amylase—Influence of Precipitant Concentration. The effectiveness of four water-miscible organic compounds for the precipitation of amylase from a mold bran extract is shown in Fig. 1. Precipitation temperatures were 20°C. for methanol (the highest temperature permitting appreciable enzyme recovery) and room temperature for the other precipitants.

At no concentration was it possible to obtain complete amylase recovery with methanol at 20°C. Recovery increased with increase in precipitant added up to 75 to 80% methanol concentration but was not improved at higher concentrations. A similar curve was obtained for ethanol but recovery was more nearly complete. It is obvious from

the curves of Fig. 1 that isopropanol was superior to the other two alcohols, since it gave higher recoveries of enzyme at lower concentrations. A further advantage for isopropanol, particularly in differential fractionation studies, is indicated by the very abrupt rise in the enzyme recovery between 50 and 55% concentration of precipitant.

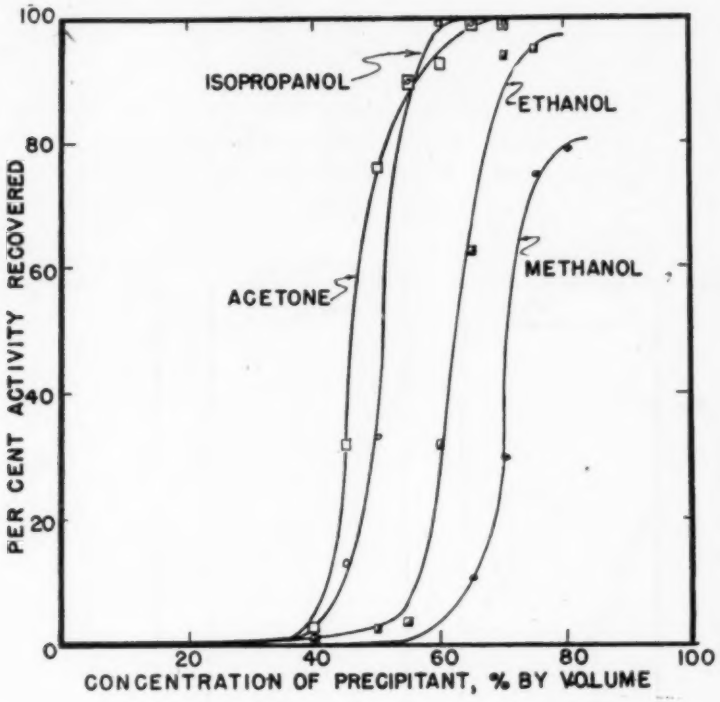

Fig. 1. Effect of precipitant concentration on recovery of amylase activity from mold bran extract.

The data shown for acetone in Fig. 1 demonstrate that it was similar to isopropanol in precipitation properties. However, acetone tended to produce a gummy, discolored precipitate of lower water solubility than that resulting from the alcohol precipitations.

Influence of Temperature. With the four organic compounds used, the recovery of amylase by precipitation was related to both precipitant concentration and temperature; the lower the temperature the lower was the precipitant concentration required for maximum recovery. This is shown in Table I for methanol and in Fig. 2 for ethanol, isopropanol, and acetone. By reducing the precipitation temperature to 0°C. it was possible to obtain enzyme recoveries of as high as 90% with methanol.

TABLE I

EFFECT OF TEMPERATURE ON THE RECOVERY OF PRECIPITATED AMYLASE WITH METHANOL AS THE PRECIPITATING AGENT

Precipitation temperature (°C.)	Per cent amylase recovered	
	75% methanol	80% methanol
0	90	92
10	82	88
20	75	79

The combined temperature-concentration effect is well illustrated in Fig. 2. For example, at 70% concentration, temperature had little effect on recovery from ethanol precipitation. However, when the precipitant concentration was reduced to 65%, temperatures below

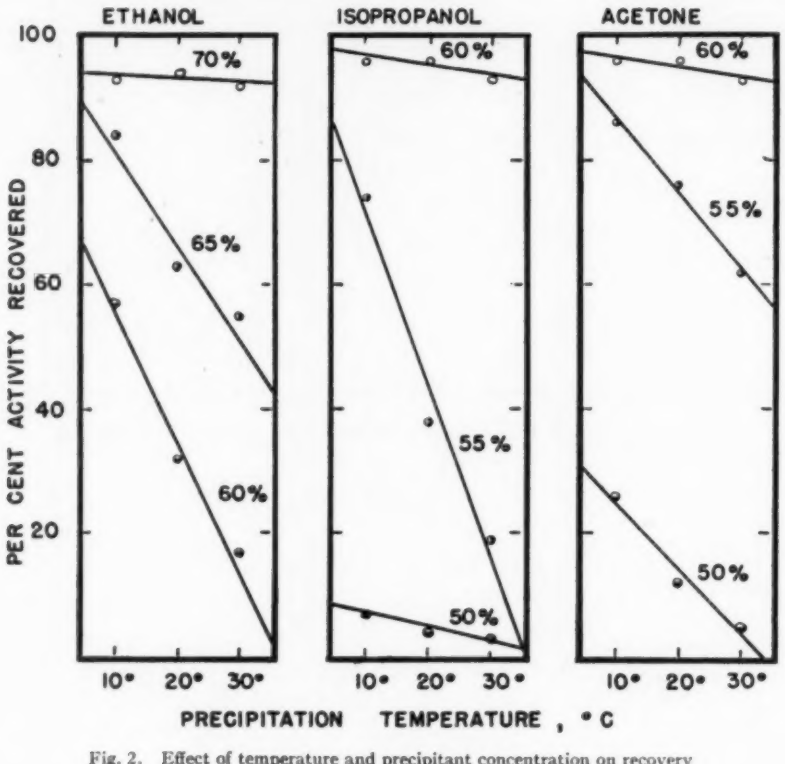

Fig. 2. Effect of temperature and precipitant concentration on recovery of amylase activity from mold bran extract.

10°C. were required to give recovery comparable to that obtained at 70%. The same trend was observed for isopropanol and acetone precipitations. In each instance a minimum precipitant concentration is indicated at which temperature variation up to 30°C. had little

effect on enzyme recovery. Below this minimum the temperature effect was very striking.

Influence of Contact with Precipitant on Precipitate Activity. It was found that enzyme recovery was influenced by the type of precipitant not only in the initial precipitation but also during the period of contact before separation and during the drying of the precipitate. The contact effect during precipitation was determined for methanol and ethanol with distilled water extracts only, and for isopropanol and acetone with both distilled water and dilute calcium chloride extracts of mold bran.

Following methanol precipitations at 20°C. a progressive decrease in enzyme recovery occurred during the time intervening before centrifugation. After 10 minutes standing 69% of the enzyme activity remained; this decreased to 63, 54, and 45% recovery respectively after an additional 1, 2, and 3 hours standing. A similar effect was noted with ethanol precipitation at 30°C. but with consistently higher recoveries of enzyme. After standing 1, 3, and 7 hours before centrifugation, recoveries were respectively 94, 88, and 83%. After 25 hours standing in contact with 70% ethanol at 30°C. only 55% of the enzyme could be recovered.

TABLE II

EFFECT OF THE PRECIPITATING AGENT AND TIME OF CONTACT WITH PRECIPITANT AT 25°C. ON THE ACTIVITY OF THE AMYLASE PRECIPITATED FROM WATER EXTRACT AND FROM 0.2% $CaCl_2$ WATER EXTRACTS OF MOLD BRAN

Time allowed to stand (min.)	Per cent amylase recovered	
	Isopropanol	Acetone
	DISTILLED WATER EXTRACT	
0	100	100
30	100	100
60	93	93
	0.2% CALCIUM CHLORIDE EXTRACT	
0	86	96
30	89	75
60	89	65

The data for isopropanol and acetone are given in Table II and demonstrate the high degree of stability of precipitated mold amylase from distilled water extracts. In contrast, the presence of calcium chloride in the extract, even at 0.2% concentration, markedly contributes to low recovery and, with acetone, to an instability of the precipitate.

The loss of enzyme activity during the period necessary for drying the precipitate was next considered. (The precipitate was allowed to

dry in the bottom of the centrifuge tube.) One series was dried for 2 hours in front of a fan before the activity was determined. Another was dried for 20 hours with no forced air circulation, and the third series was centrifuged and the activity of the precipitate determined immediately. The data shown in Table III indicate more danger of

TABLE III

EFFECT OF DRYING ON THE AMYLASE ACTIVITY FOLLOWING PRECIPITATION WITH 75% METHANOL, 70% ETHANOL, AND 60% ISOPROPANOL AND ACETONE (30°C.)

Time allowed to dry (hrs.)	Per cent amylase recovered			
	75% methanol	70% ethanol	60% isopropanol [1]	60% acetone
0	70	100	88	100
2	69	100	85	100
20	48	83	91	94

[1] Low recovery presumably due to factors other than drying.

loss in enzyme activity from drying methanol and ethanol precipitates than with the other two precipitants.

Influence of Hydrogen-ion Concentration. The difficulty of obtaining complete recovery with methanol and the apparent detrimental effect on the activity of the precipitate discouraged any additional work with this compound, and only ethanol, isopropanol, and acetone were investigated further.

The influence of the hydrogen-ion concentration of the mold bran extract on the recovery of enzyme by ethanol, isopropanol, and acetone precipitation was determined. The pH values were adjusted with hydrochloric acid or sodium hydroxide before addition of the precipitating agent. The concentration of precipitants used was 70% for ethanol, and 60% for both isopropanol and acetone. In all instances the precipitation temperature was 25°C. The data were recorded in terms of per cent enzyme activity recovered at the various pH values and are shown graphically in Fig. 3.

As may be seen from Fig. 3 the optimum hydrogen-ion concentration for ethanol precipitation was between pH 6.0 and 6.5. With isopropanol and acetone the optimum was somewhat lower, being between pH 5.5 and 6.0; either above or below the optimum pH value there was a decrease in enzyme recovery, being very pronounced on the acid side.[4]

[4] It is appreciated that at least part of the low recoveries on the acid and alkaline sides of the optimum pH value may be attributed to enzyme inactivation. The data of Fig. 3 show the overall effects.

With ethanol precipitation it was observed that with an increase in pH value up to and through the optimum the precipitates became more dense and flocculent. Precipitates formed at pH values in the region of 2.5 to 3.5 remained in suspension and could be separated from the media only by centrifuging. With isopropanol and acetone no effect of pH on rate of precipitate settling was observed—there was very little variation over the pH ranges used, and all precipitates settled slowly.

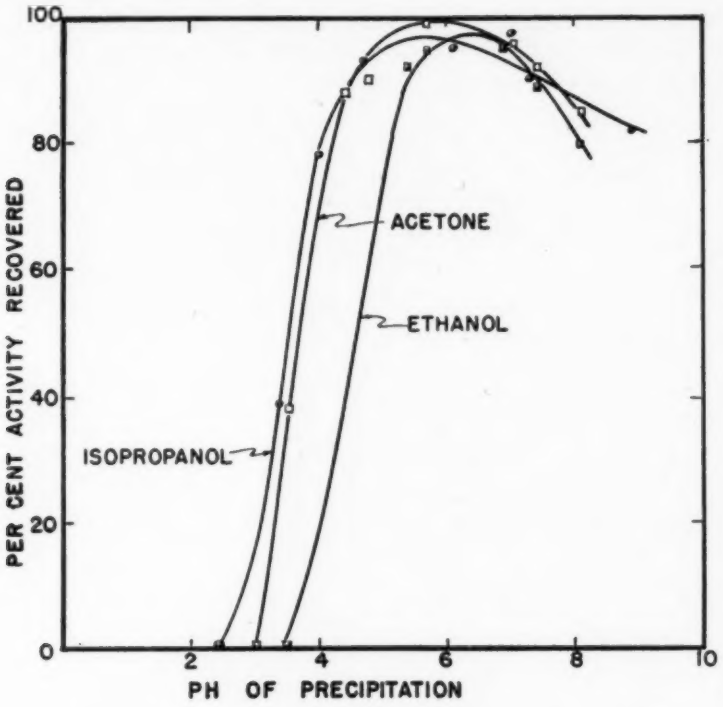

Fig. 3. Effect of hydrogen-ion concentration on recovery of amylase activity from mold bran extract.

The data shown by the curves of Fig. 3 were all obtained using distilled water extracts of mold bran. Precipitations with isopropanol were also carried out over a wide pH range, using a 0.2 per cent calcium chloride extract. The same pH optimum was found as for a distilled water extract, i.e., pH 5.5 to 6.0. However, a notable difference was observed in the rate of settling of the precipitates. In this case, as with ethanol, precipitates formed on the acid side of the optimum tended to remain in suspension while those formed near neutrality settled rapidly.

Influence of Quality and Quantity of Ions Present. There are two kinds of salt addition which might be made in an enzyme precipitation study. Certain techniques depend solely on the salt to precipitate the enzyme-active principal. Other methods incorporate a small amount of some salt as an aid to help carry down the active principal when an organic compound is used as the precipitating agent. Precipitates from distilled water extracts without salt addition were not easily filtered and were somewhat difficult to handle. The study of the effect of salts on precipitation was undertaken to find an aid in improving the physical characteristics of the precipitate, i.e., rate of flocculation and settling, filterability, and ease of powdering, without causing any decrease in activity of enzyme recovered.

TABLE IV

PRECIPITATION OF AMYLASE IN THE PRESENCE OF LOW CONCENTRATION OF VARIOUS SALTS

(Salt concentrations: with isopropanol, 0.04 *N*, with ethanol and acetone, 0.10 *N*) [1]

Salt	Per cent amylase recovered		
	70% ethanol	60% isopropanol	60% acetone
NaCl	97	89	100
KCl	97	89	—
$BaCl_2$	83	87	82
$CaCl_2$	73	84	83
K_2SO_4	—	—	95
$(NH_4)_2SO_4$	—	96	—
Na_2SO_4	98	—	93
KH_2PO_4	—	91	—
K_2HPO_4	—	95	—
$Na_2HPO_4 \cdot 12H_2O$	93	—	93
$NaH_2PO_4 \cdot H_2O$	93	—	—
$MgSO_4 \cdot 7H_2O$	93	—	93
$MgCl_2 \cdot 6H_2O$	—	—	95
$Al_2(SO_4)_3 \cdot K_2SO_4 \cdot 24H_2O$	—	0	—
$Fe_2(SO_4)_3 \cdot (NH_4)_2SO_4 \cdot 24H_2O$	—	0	—

[1] The addition of certain salts might influence enzyme recovery by producing changes in hydrogen-ion concentration. However, this is believed unimportant because of the strong buffering action present in the mold bran extract and the low concentration of salts used. Also aluminum and iron are known to have inactivation potentialities.

The effect of various salts on precipitation by ethanol, isopropanol, and acetone was investigated, in each instance the salt being added before the precipitant to give 0.1 *N* concentration for ethanol and acetone and 0.04 *N* for isopropanol. The data are given in Table IV. It will be noted that the same salts were not used with all precipitants.

With the exception of calcium chloride and barium chloride, the salts studied with ethanol precipitation had little influence on the

recovery of enzyme. However, a pronounced influence on the physical characteristics was apparent: the phosphates tended to result in gummy precipitates; calcium and barium chloride gave flocculent, rapidly settling precipitates; and the other salts gave precipitates that settled rapidly but incompletely, leaving a cloudy supernatant liquid. The excellent physical characteristics of the precipitate formed in the presence of either calcium or barium chloride was a desirable feature but was nullified by the loss of enzyme activity.

In the isopropanol precipitation studies on the effect of different ions, several common salts were chosen to include representatives of mono-, di-, and trivalent cations and anions. The data of Table IV show that with an increase in the valence of the cation the activity of the precipitate decreased. The ferric and aluminum salts produced a voluminous precipitate which had no activity. Calcium and barium produced a lesser amount of precipitate. The monovalent cations produced the least precipitate but with the greatest activity. Again the separation obtained in the presence of the divalent cations seemed to be preferable and more complete than that obtained with the monovalent cations.

As far as the anions are concerned, the grouping by valence was not so distinct. Based on the activity of the precipitates produced from extracts, an anion series may be arranged as follows: phosphate, acetate, sulfate, chloride, nitrate, oxalate, citrate, tartrate, and sulfite. A variation of about 20% in enzyme activity existed between the first and last of this series.

The results with acetone, Table IV, support evidence gained from studies with the other precipitants showing that calcium and barium ions have a detrimental effect on the enzyme recovery. Further, the sulfate salts had a very pronounced effect on the physical characteristics of the acetone-produced precipitates from mold bran extracts. With magnesium sulfate in a concentration of 0.10 *N* the precipitate appeared as a semiliquid, sticky mass after centrifugation. If disodium phosphate was added to the aliquot containing magnesium sulfate, the precipitate appeared as a white floc that settled rather rapidly, leaving a clear supernatant liquid. In the tubes containing solutions of calcium and barium ions the precipitates had much the same appearance as those which contained the magnesium sulfate and disodium phosphate.

A brief study was made of the effect of concentration of calcium chloride and sodium chloride, when added to distilled water extracts, on the activity of the precipitates from 70% ethanol. The results are given in Fig. 4 and indicate a very detrimental effect of the calcium ion as compared to the sodium ion.

Additional information was obtained on the effect of calcium chloride concentration by a comparison of the tolerance of the three precipitating agents, ethanol, isopropanol, and acetone, to the presence of this salt. Aliquots of extract were adjusted to 0.10 and 0.20 *N*

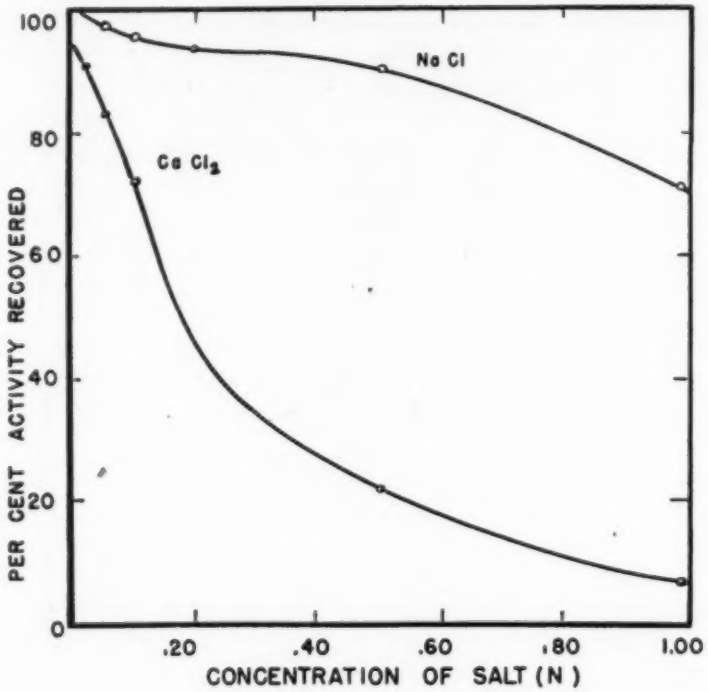

Fig. 4. Effect of salt concentration contrasting NaCl and $CaCl_2$ on the recovery of amylase activity from mold bran extract.

calcium chloride before being precipitated with 70% ethanol or 60% isopropanol or acetone. As shown in Fig. 5, when the activities of the precipitates thus formed were determined, it became evident that isopropanol precipitation was less influenced by the presence of excess calcium ions than precipitations by either of the other agents.

To isolate the individual salt effects, aliquots of an extract were dialyzed against various media and their behavior determined after dialysis. Four aliquots of extract were dialyzed respectively against distilled water, 0.043 *N* sodium chloride, 0.060 *N* calcium chloride, and 0.060 *N* disodium phosphate solutions for 48 hours at 10°C. After dialysis the extracts were adjusted to their original volumes and the activity of each was compared with that of an original extract. No loss of activity was observed in any of the solutions, or in a control allowed to stand at the same temperature without dialysis.

Aliquots of each of these dialyzed solutions were precipitated with 70% ethanol. For comparison, aliquots of an undialyzed extract, to which had been added equal amounts of salt, were precipitated under the same conditions. The results are given in Table V and demon-

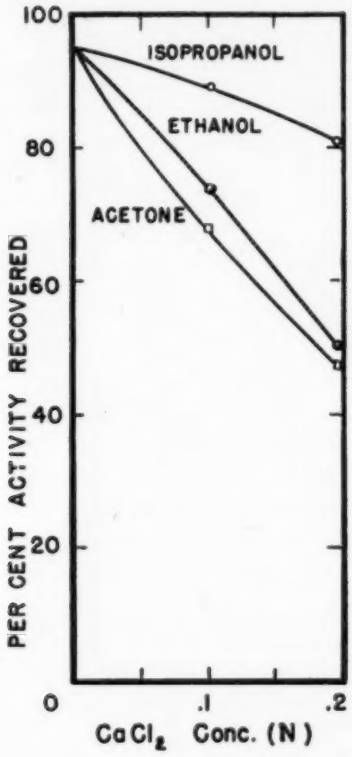

Fig. 5. Effect of calcium ion concentration on recovery of amylase activity.

strate that the presence of some salt is imperative to the recovery of enzyme activity in the precipitate. However, none of the salts used in the dialyzing solutions gave recoveries comparable to that obtained with the undialyzed extracts. The extracts dialyzed against distilled water produced only milkiness when ethanol was added, and when these were centrifuged very little precipitate settled out. The other dialyzed solutions produced small amounts of precipitate with cloudy supernatant liquids, indicating incomplete separation of the precipitated phase.

The foregoing results indicated that there was some dialyzable substance extracted from the bran, which served as an aid in recovering an active precipitate. To determine the source of this precipitating aid, i.e., whether it is in the natural wheat bran or is something formed or

added in the production of the mold bran, a sample of mold bran and a sample of ordinary wheat bran were extracted, filtered, and precipitated under the same conditions. The wheat bran extract gave a precipitate with physical characteristics similar in appearance to those of the precipitate from mold bran.

TABLE V

PRECIPITATION OF AMYLASE BY 70% ETHANOL FROM MOLD BRAN EXTRACTS DIALYZED AGAINST DISTILLED WATER AND AGAINST SALT SOLUTIONS

Dialysis medium	Salt concentration (*N*)	Per cent amylase recovered	
		Dialyzed solution	Undialyzed solution
Distilled water	—	21	100
NaCl	0.043	89	100
$CaCl_2$	0.060	89	83
$Na_2HPO_4 \cdot 12H_2O$	0.060	59	100

An analysis of wheat bran reported by Bailey (1) indicates a high content of the four elements, phosphorus, magnesium, calcium, and potassium. These elements were considered singly and in combination to determine their effect on the appearance of the precipitate.

A quantity of mold bran extract was dialyzed for 24 hours against distilled water. Aliquots of this extract were adjusted to various concentrations with various salts and precipitated with 70% ethanol. Any one of the salts studied aided in recovery of the enzyme. However, as may be seen in Table VI, some appeared to cause an increasing loss of enzyme with increases in concentration. Only the magnesium

TABLE VI

RECOVERY OF AMYLASE BY ETHANOL PRECIPITATION IN DIALYZED MOLD BRAN EXTRACTS WITH ADDED SALTS

Salt added	Per cent amylase recovered		
	0.10 *N* salt	0.50 *N* salt	
No salt added	less than 5	—	
NaCl	97	97	
KCl	93	93	
$MgCl_2 \cdot 6H_2O$	90	75	
$CaCl_2$	52	less than 5	
K_2HPO_4	13	30	
	0.012 *N* salt		
$CaCl_2$			
$Na_2HPO_4 \cdot 12H_2O$	69		
	0.023 *N* salt	0.036 *N* salt	0.092 *N* salt
$MgCl_2 \cdot 6H_2O$			
$Na_2HPO_4 \cdot 12H_2O$	94	98	96

chloride-disodium phosphate combination produced a white, flocculent precipitate characteristic of undialyzed solutions. All other salts or combinations produced milky, slow-settling precipitates.

In an investigation to determine the optimum concentration of magnesium sulfate and disodium phosphate required for precipitation, no sharp crest occurred. However, concentrations of 0.028 *N* and greater left increasing amounts of insoluble residue when the precipitates were redissolved. Magnesium sulfate was substituted for magnesium chloride because of its greater ease of handling and because no difference was found in the effectiveness of the two salts as precipitating aids with ethanol employed as the precipitant. Variations in the concentration of magnesium sulfate and disodium phosphate from 0.019 *N* to 0.046 *N* had no effect on the per cent recovery of enzyme.

Studies were conducted to determine the effect of varying ratios of the magnesium ion and monohydrogen phosphate ion on the activity of the precipitate, and the data obtained are given in Table VII. An

TABLE VII

EFFECT OF VARIABLE $(Mg)^{++}/(HPO_4)^{--}$ RATIO AND TIME OF STANDING ON THE AMYLASE ACTIVITY OF THE PRECIPITATE PRODUCED WITH 70% (BY VOLUME) ETHANOL

Concentration (*N*) $MgSO_4 \cdot 7H_2O$	Concentration (*N*) $Na_2HPO_4 \cdot 12H_2O$	$(Mg)^{++}/(HPO_4)^{--}$ ratio	Per cent amylase recovered	
			10 min.	3 hrs.
0.047	0.019	2.6	90	62
0.038	0.019	2.0	—	72
0.029	0.019	1.5	90	78
0.019	0.019	1.0	—	87
0.019	0.029	0.66	98	92
0.019	0.038	0.50	—	92
0.019	0.056	0.34	98	92
0.019	0.073	0.26	—	96

excess of magnesium was detrimental to the activity of the precipitate. This fact supports evidence of loss caused by excess of the divalent calcium and barium ions when salts of these ions were studied as precipitation aids. When the element of time was introduced along with the factor of ionic ratio, it was demonstrated that an excess of monohydrogen phosphate ion stabilized the precipitate. One series of precipitations was made and allowed to stand for 3 hours before being centrifuged, while another was centrifuged immediately after precipitation. With an increase in the $(Mg)^{++}/(HPO_4)^{--}$ ratio from 0.34 to 1.0 the rate of settling increased and the clarity of the supernatant liquid decreased. Ratios below 0.34 produced a cloudy supernatant liquid and rapid settling.

The tendency of acetone to produce discolored, gummy, and water-insoluble precipitates removed this precipitant from those considered for additional investigation.

Batch Precipitation. Having established that ethanol and isopropanol were the more promising precipitating agents, an effort was made to prepare a quantity of the enzyme concentrate with each of these precipitants. Attempts to dry precipitates from ethanol were rewarded with isolates of high activity and good physical characteristics except for water solubility. By using a concentration of 0.25% magnesium sulfate and 0.72% disodium phosphate, by weight, in the enzyme extract, a white to buff precipitate was formed that had 10 times the activity of the original mold bran. The precipitate was separated by centrifugation, resuspended in 95% ethanol, and filtered. Approximately 68% yield was obtained, based on the total enzyme content of the mold bran.

In the batch precipitations with isopropanol similar results were obtained. In Table VIII are listed the data on four trials at isolating and drying concentrates from isopropanol precipitation.

TABLE VIII

RECOVERY OF AMYLASE IN PRECIPITATES FORMED WITH ISOPROPANOL

Batch	Volume of extract	Precipitate activity *dT* of 0.005 g.[1]	Yield recovery	
	ml.	*min.*	*g.*	%
1	100	40.5	1.94	92
2	100	9.0	0.39	71
3	100	13.0	0.68	86
4	1000	10.5	6.45	74

[1] "dT" signifies dextrinization time.

In each case 0.25% magnesium sulfate and 0.50% disodium phosphate, by weight, were added to the extract before precipitation with 60% isopropanol. The residue after centrifugation was dispersed in a volume of isopropanol one-fifth the original volume of the extract.

The precipitates formed were separated principally by centrifugation since their physical properties did not permit easy filtration either by gravity or suction. Either a supercentrifuge or large capacity centrifuge was found necessary to obtain satisfactory separation.

Properties of Precipitates. When comparing the activities of the two types of precipitates, one from ethanol and the other from isopropanol, it was found that the one resulting from isopropanol was somewhat more active. For example, 0.05 g. of the mold bran gave a dextrinizing time of 13 minutes; 0.005 g. of the ethanol precipitate gave an activity represented by the same dextrinizing time, 13.0

minutes; and 0.005 g. of the isopropanol precipitate gave a dextrinizing activity of 10.5 minutes.

In evaluating and comparing the saccharifying power of mold bran and concentrates prepared from it, extracts of the above three sources were adjusted to the same dextrinizing activity and then equal amounts (dT—20 minutes) of the extracts added to a 2% starch substrate plus yeast nutrients and allowed to digest for 1 hour at 30°C. At the end of this hour 0.5 g. of compressed yeast was added to the fermentation cup and the fermentation was followed manometrically. Readings were taken at 1, 3, 5, 10, 21, and 22 hours. The data in Table IX

TABLE IX

SACCHARIFYING POWER OF AMYLASE EXTRACTS FROM VARIOUS SOURCES

Enzyme source	Wt. equal (mg.) dT—20 min.	Pressure—mm. mercury [1]					
		1 hr.	3 hrs.	5 hrs.	10 hrs.	21 hrs.	22 hrs.
FUNGAL CONCENTRATES							
Commercial A	1.3	17	81	147	251	327	336
Commercial B	1.0	15	80	138	270	323	329
Commercial C	0.76	17	67	144	276	331	338
Ethanol precipitate	3.5	14	59	123	272	339	341
Isopropanol precipitate	2.6	15	65	140	293	383	387
FUNGAL BRANS							
A	6.1	16	67	145	281	361	364
B	19.7	13	71	153	290	363	366
C	12.5	14	72	153	280	343	346
D	31.4	15	63	138	296	390	390

[1] These figures give an indication of the rate of fermentable sugar production under fermentation conditions.

include, for comparison, information on the other mold brans and fungal concentrates.

It will be noted that all of the enzyme sources listed in Table IX gave approximately the same fermentation pattern. However, the commercial mold bran D and the isopropanol precipitate gave greater total conversion than any other of the enzyme sources, as indicated by the readings at 22 hours. It also should be noted that the isopropanol precipitate was superior to the ethanol precipitate in total starch conversion.

Discussion

The data herewith presented show conclusively that an active precipitate may readily be obtained from mold bran extracts by the

use of a water-miscible organic compound. However, to recover high yields of enzyme in a precipitate qualitatively similar enzymatically to the mold bran and with desirable physical characteristics, the precipitating conditions should be adjusted with some care. To satisfy these conditions and, in addition, to give greatest enzyme recovery at lowest concentration of precipitating agent, isopropanol was the compound of choice among those tested. In addition isopropanol precipitation proved to have a low degree of sensitivity to the presence of otherwise undesirable salts.

The influence of certain ions on the recovery of enzyme and on the physical characteristics of the precipitate has considerable significance in commercial application. The presence of certain bivalent cations should be avoided. This applies particularly to the commonly occurring calcium ions. On the other hand, the presence of magnesium ions is desirable, but only if in conjunction with balancing phosphate ions. In fact, the presence of adequate magnesium and phosphate ions appeared to be a prerequisite to satisfactory precipitation. Customarily, these should be present in amounts supplementary to the quantities already present in the mold bran extract.

Since adequate precipitation could not be obtained with extracts dialyzed free of salts, it appears that the problems of precipitation are mainly those related to these salts. Satisfactory precipitation with a water-miscible organic compound proved to depend on the adjustment of conditions such that a good precipitate of magnesium phosphate was obtained, which in turn carried with it the desired enzyme in a concentrated form.

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HYDROLYSIS OF THE AMYLOPECTINS FROM VARIOUS STARCHES WITH BETA-AMYLASE [1]

JOHN E. HODGE, EDNA M. MONTGOMERY, and G. E. HILBERT [2]

ABSTRACT

The amylopectin or branched-chain fractions of corn, wheat, white potato, sweet potato, and tapioca starches were isolated by removal of the amylose or linear fraction after its precipitation with *n*-butanol, then degraded by beta-amylase to the limit dextrins. The extent of conversion to maltose, yields of limit dextrins and crystalline maltose hydrate, phosphorus contents, alkali labilities, iodine sorptions, specific optical rotations, and properties of the triacetyl derivatives were determined.

The branched-chain fractions from the different starches were alike in extent of conversion by beta-amylase, alkali lability, specific optical rotation, and in some properties of the acetates. They differed in phosphorus content, the nature of the phosphorus present, and iodine sorption. The root and tuber limit dextrins retained phosphorus, whereas the cereal limit dextrins did not. Evidence was found which was interpreted as indicating the existence in some starches, particularly corn and sweet potato, of a fraction intermediate in the extent of branching between linear amylose and the average for branched amylopectin.

References are made in recent literature to the uncertainty that exists as to whether the amylopectins of starches from different plant

[1] Manuscript received August 20, 1947. Presented at the Annual Meeting, May, 1947.
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sources are alike or different, and the need for further investigations on the structure of amylopectin by means of enzymic degradation has been indicated.

In studies of the structure of the amylopectin or branched-chain fraction of starch by enzymic degradation, it is desirable that the amylopectin first be isolated. Much of the work in the past has been performed on whole starch, usually degraded soluble starch (5, 7, 8, 9, 13). In other studies (4, 14, 15, 17, 18, 23) attempts were made to isolate the amylopectin fraction, but the methods of fractionation used (hot water extraction and electrodialysis) generally have yielded amylopectins of doubtful purity. In recent years Schoch (24) has developed a more efficient method for separating the linear from the non-linear molecules of starch, involving the crystallization of the linear fraction as a complex with *n*-butanol; but only Kerr (11) has applied the method to isolate branched material for degradation with beta-amylase. Kerr's work was confined to branched-chain fractions from corn starch. The waxy starches occur in nature virtually free from linear molecules. Their degradation by beta-amylase also has been studied (3, 16, 17, 19).

The work reported here compares the amylopectin fractions of corn, wheat, white potato, sweet potato, and tapioca starches by determining the extent of their conversion to maltose by beta-amylase. The amylopectins and corresponding limit dextrins were examined for alkali lability, specific optical rotation, properties of the acetate derivatives, phosphorus content, iodine sorption, and anomalous linkage content as shown by the degradation of the limit dextrin triacetates in hydrogen bromide-acetyl bromide-acetic acid reagent (10). The starches were not autoclaved before fractionation, the amylopectins were isolated by precipitation of the amylose fractions as crystalline complexes with *n*-butanol, and the preparation of beta-amylase from ungerminated wheat was free from alpha-amylase and maltase.

Materials and Methods

Preparation of Amylopectins. Most of the starches selected were isolated in this laboratory under conditions which gave products of high purity with a minimum degree of alteration from the native state of the granules. Only distilled water was used for steeping, except for the wheat which required a 0.1% sulfur dioxide steep. The tapioca, corn II, and sweet potato I starches were high-grade commercial samples. The corn and wheat starches were defatted by methanol extraction in a Soxhlet apparatus, whereas the root and tuber starches contained 0.2% or less of methanol-extractable substances at the outset and were not defatted.

The method of Schoch (24) for fractionating starch with *n*-butanol has been altered in this laboratory by Karjala, Wolff, and Olds (unpublished experiments) to avoid degrading the starch by autoclaving. Starch was pretreated with liquid ammonia at −35°C. to swell and partially disorganize the granular structure. Mixing ethanol with the suspension of starch in liquid ammonia allowed isolation of the starch, after evaporation of the ammonia, in a granular state. The dried starch was reactive toward acetylation, showed no crosses by polarized light, and could be dispersed effectively in water saturated with *n*-butanol at 90°C. Following Schoch's method the linear fraction was crystallized in Dewar vessels and removed from the branched material by the continuous supercentrifuge. One additional pass through the supercentrifuge produced a homogeneous amylopectin solution (2–3% concentration, pH 6.5 ± 0.2). After distillation *in vacuo* below 50°C. a nearly clear, butanol-free paste remained, 8–10% amylopectin by weight. The concentration was determined by drying weighed samples of paste to constant weight under an infra-red lamp at 120°C.

The major part of this paste was used for hydrolysis by beta-amylase; the remainder was precipitated in nine parts of ethanol in a Waring Blendor to obtain a fluffy powder easily resoluble in water. White potato starch II was dispersed for fractionation without pretreatment with liquid ammonia by simply adding a suspension of it in butanol to stirred butanol-water at 90°C. The yields, 66% for corn, 72–75% for white potato, sweet potato, and tapioca amylopectins, were close to the amounts expected from the reported amylose contents of these starches (1, 24), considering that 3–6% of butanol-water soluble substance in the starch was precipitated with the amylose fraction.

In addition to the amylopectins isolated as described above, a fraction of the amylopectin of corn starch II was separated from the crude amylose-butanol precipitate. The crude amylose complex (approximately 100 g. dry weight) was dissolved in 9 liters of water saturated with *n*-butanol at 90°C. and autoclaved for 1 hour at pH 6.7 (initial) to 6.3 (final). After 2 days of slow cooling with stirring, the recrystallized amylose-butanol complex was removed cleanly by supercentrifugation, yielding 80 g. of purified amylose (iodine sorption: 194 mg./g.). The centrifugate was concentrated *in vacuo* to a 5% paste. A part of the paste was precipitated in ethanol and the remainder was hydrolyzed with beta-amylase.

Preparation of Beta-Amylase. The method was essentially that of Van Klinkenberg (29) as applied by Hanes (6) to barley and Haworth *et al.* (8) to wheat. The continuous supercentrifuge was used to separate the 50% (by volume) cold aqueous ethanol-soluble, 80% cold

aqueous ethanol-insoluble fraction from finely ground whole wheat (Trumbull variety, soft red winter wheat; grown at Wooster, Ohio, 1944 crop). The dry powder obtained (35 g. from 3 kg. wheat) was extracted with 2 liters of water in portions to form a solution of the enzyme. The filtered, unbuffered solution, preserved with toluene and thymol and kept below 5°C., was used as a source of beta-amylase for all the hydrolyses. Filtering before each use removed an amorphous precipitate which formed in the solution on standing. The pH of the solution increased from 6.0 to 6.7 in the first 6 weeks but remained constant thereafter. Seven months after preparation, at the end of the experiments, the strength per gram of the solution was 1.15 Kneen-Sandstedt beta-amylase units (12).

The beta-amylase preparation was free from alpha-amylase because the percentage of amylopectin hydrolyzed in the presence of a large excess of beta-amylase reached a limit that was constant after 24, 30, 48, and 60 hours of incubation at 36°C., pH 6.5. The method of Olson, Evans, and Dickson (20) for detecting alpha-amylase in beta-amylase preparations gave negative results on other solutions prepared in identical manner from the same wheat. Maltase was absent because the optical rotation and reducing power of a solution of maltose and the enzyme were constant. The reducing power of the enzyme solution alone toward Fehling's solution was 0.6 mg. maltose equivalent per gram initially, and decreased with aging.

Hydrolysis of Amylopectins with Beta-Amylase. To facilitate the quantitative isolation of limit dextrin and maltose hydrate, no buffer salts were added to the substrate. The pH remained constant in the range 6.4–6.9 throughout all the hydrolyses.

Example: One kg. of unbuffered paste containing 95.0 g. of amylopectin was treated with 200 g. of the enzyme solution. After mixing 2–3 ml. of toluene into the paste, the flask was immersed in a constant temperature bath at 36°C. and shaken at intervals. Weighed samples were taken for reducing sugar determinations at 6, 18, and 24 hours. After 24 hours, 25 g. additional enzyme solution was added, and the flask was kept at 36°C. for another day. At 30 and 48 hours the reducing power was again determined; in no case did the conversion continue after the second addition of enzyme. The rate of hydrolysis in a typical case (wheat amylopectin), as measured by the reducing action of the maltose formed, follows: In 2, 6, 24, 30, and 48 hours at 36°C. (C 7.9%, pH 6.7), 51.0, 55.0, 55.8, 55.4, and 55.5%, respectively, of the amylopectin was found converted to maltose.

A duplicate series of hydrolyses was run at nearly the same concentrations, but on a smaller scale, by dissolving the precipitated amylopectins (20 g.) in water (180 g.) at 80°C., diluting to 9% concentration,

cooling, and adding 30 g. enzyme solution. The extents of hydrolysis and recoveries of limit dextrin and maltose were virtually the same as obtained in the first series.

Isolation of Limit Dextrins and Maltose Hydrate. The limit dextrins were precipitated from the hydrolyzates by adding ethanol to 60% concentration by volume. To obtain complete precipitation of the potato dextrins, approximately 1 g. of sodium chloride was added. The gummy mass of dextrin was squeezed, redissolved in 10–12 parts water, and precipitated in the Waring Blendor in the ratio one part paste to nine parts absolute ethanol. By frequently rinsing down the sides of the Blendor, the precipitating jars, and the Buchner funnel with ethanol, no significant losses occurred. The dextrin was dried in a vacuum desiccator, then at 80°C. in the vacuum oven. As the dextrin was weighed to determine the yield, a sample was taken for a moisture determination. The limit dextrin was purified by redissolving in hot water, precipitating twice at 50% ethanol concentration and a third time in absolute ethanol as before. The reducing powers of the purified dextrins were approximately 0.1% maltose equivalent; the nitrogen contents were 0.05–0.07% compared to 0.01–0.02% for the amylopectins.

For isolation of the maltose hydrate, the alcoholic liquors from the first and second precipitations were filtered, concentrated *in vacuo* below 40°C., refiltered, and further concentrated to a syrup. Crystallization was effected at 65 to 75% ethanol concentration with seeding. The mother liquors were concentrated and two additional crops of crystals were obtained. The yields of maltose hydrate were calculated from the maltose content of the crystalline fractions as determined by their optical rotation, on the assumption that the impurities were optically inactive. The purities of the first crops were usually higher than 98%. Highly purified beta-maltose hydrate was prepared for standardization of the reducing sugar method by recrystallizing three times from 60–65% ethanol and drying over calcium chloride in a vacuum desiccator. The product was finally dried at 50°C., 1 mm. pressure, for 5 hours in a vacuum oven. $[\alpha]_D^{25}$ after 5 minutes in solution, + 114°; final, + 130.4° (C 4.0, H_2O). "Melting" range: 121°–125°C. (varying within these limits with degree of subdivision and rate of heating); the opaque "melt" frothed at 140°–150°C. and was then clear and colorless. Water content: 5.01%; calculated for the monohydrate 5.00%. On exposure at 40–50% relative humidity, the water content increased to 5.10 ± 0.03%. Water was determined by drying 1-g. samples to constant weight in an Abderhalden apparatus over boiling toluene (pressure less than 1 mm.); the results were confirmed by Karl Fisher reagent.

Acetylation of Limit Dextrins. Five g. of the purified limit dextrin were mechanically stirred with 50 ml. dry pyridine in a flask protected from atmospheric moisture. A mixture of 30 ml. acetic anhydride (three times theory) in 20 ml. pyridine was added, and the mixture was stirred 6 hours in a bath at 100°C. A double quantity of the acetic anhydride-pyridine mixture was necessary in the acetylation of the potato limit dextrins because they formed stiff gels that were difficult to stir. The limit dextrin acetate was precipitated in ethanol using the Waring Blendor, washed thoroughly in 50% aqueous ethanol, and dried *in vacuo* at 25°C. and 100°C. This acetylation procedure was essentially that of Whistler, Jeanes, and Hilbert (31). The acetyl contents were 44.8 ± 0.3% for all but sweet potato I acetate which was 44.2% acetyl; the theoretical amount for complete acetylation is 44.8%.

Analytical Methods. The maltose in the hydrolyzates was determined by the Munson-Walker oxidation with Fehling's solution. The reduced copper was determined without filtration by the iodometric "cuprous" titration of Shaffer and Hartmann (27). Samples of hydrolyzate were taken to contain approximately 100 mg. maltose hydrate. Walker's table for maltose hydrate (30) was found to give results correct within 1.5% (average deviation ± 0.7%) when 100 mg. of the purified maltose hydrate was used as a standard. The reducing power contributed by the added enzyme solution was less than the probable error of the maltose determination.

The analytical procedure for phosphorus was that of Truog and Meyer (28). Alkali lability was determined by the method of Schoch and Jensen (25), except that the bottles were heated in steam baths. To determine iodine sorption, 40 mg. of the starch fraction in 100 ml. solution (0.05 *N* with potassium iodide and potassium chloride) was titrated with 0.001 *N* iodine (1, 32). From a graph of the titration, the point of inflection was determined. By subtracting from the volume of iodine solution required to reach the point of inflection that volume used in the blank titration to reach the same EMF as the point of inflection, the amount of iodine sorbed by each starch fraction was estimated. No distinction was made between the iodine actually in complex formation and that sorbed mechanically. The optical rotations of the limit dextrin acetates in the hydrogen bromide-acetic acid-acetyl bromide reagent were determined exactly as prescribed by Jeanes and Hilbert (10).

Results [3] and Discussion

The average results for the hydrolyses are given in Table I. The percentages of amylopectin hydrolyzed to maltose hydrate (column 2)

[3] All numerical results in this paper are calculated to the dry weight of the starch fractions.

were calculated from the constant values of the reducing powers of the hydrolyzates. The sums of columns 2 and 3 in all cases fall within 100 ± 1%.

TABLE I

HYDROLYSIS OF AMYLOPECTINS WITH BETA-AMYLASE—CONCENTRATION OF AMYLOPECTIN 6–8%, pH 6.4–6.9, TEMPERATURE 36°C., TIME 48 HOURS

Source of amylopectin	Hydrolysis to maltose hydrate (by copper reduction) (2)	Limit dextrin isolated (dry basis) (3)	Maltose hydrate isolated (4)
	% of theory	%	% [1]
Corn I	57	43.5	96
Corn II [2]	56.5	44.5	—
Wheat	55.5	45	91
Sweet potato I	54	45	94
Sweet potato II	54	46	—
White potato I	54	47	95
White potato II	53.5	46	91
White potato IIa [3]	53.5	47	—
Tapioca	53	47.5	95

[1] Per cent of the amount expected, calculated from the limit dextrin isolated.
[2] Corn amylopectin II was isolated from crude amylose-butanol precipitate by recrystallizing the amylose and recovering the solubles.
[3] Potato starch IIa was not treated with liquid ammonia before fractionation, otherwise the same as II.

The root and tuber amylopectins (tapioca, sweet potato, and white potato) were hydrolyzed by beta-amylase to the same extent, 53–54%; the cereal amylopectins (wheat and corn) were hydrolyzed to a slightly greater extent, 55–57%. However, the iodine sorption numbers of the cereal amylopectins were relatively high and decreased appreciably after the action of beta-amylase (Table IV), suggesting the contamination of the cereal amylopectins with linear or nearly linear molecules. If a correction is made on the assumption that the difference between the iodine titers of the amylopectin and limit dextrin is a measure of the contamination with linear molecules of amylose, the corrected percentages of hydrolysis for the wheat and corn amylopectins would be 54 and 55%, respectively. Then the amylopectins from various plant sources would have approximately the same limit of conversion by beta-amylase.

The results of the alkali lability determinations in Table II show no significant differences among the amylopectins and limit dextrins. The alkali numbers of the limit dextrins are approximately double those of the amylopectins, lending support to the view (11, 26) that the alkali lability of a starch fraction is dependent on the proportion of reducing end groups and varies inversely with the molecular weight if the linear branches containing the reducing end groups are of the same length.

The specific optical rotations of the amylopectins and limit dextrins were determined at 1% concentration in (a) water, (b) 33% aqueous calcium chloride, and (c) 5% aqueous sodium hydroxide. The specific rotations for all varieties were the same (± 1°) in each solvent. For the amylopectins they were $[\alpha]_D^{25}$ (a) +198°, (b) +204°, (c) +162°; and for the limit dextrins (a) +197°, (b) +201°, (c) +162°. These values are much higher than those obtained by Haworth and co-workers (8) for the beta-amylase limit dextrin of soluble starch.

TABLE II

ALKALI LABILITY NUMBERS OF AMYLOPECTINS AND LIMIT DEXTRINS

Source of amylopectin	Amylopectin	Limit dextrin	Ratio
Corn I	2.5	5.2	2.1
Corn II [1]	5.7	5.1	—
Wheat	2.9	4.3	1.5
Sweet potato I	2.6	4.6	1.8
White potato I	1.9	3.2	1.7
White potato II	1.4	4.1	2.9
Tapioca	1.5	4.1	2.7

[1] Corn amylopectin II was isolated from crude amylose-butanol precipitate by recrystallizing the amylose and recovering the solubles.

The triacetates of the limit dextrins from corn, wheat, and tapioca amylopectins were soluble in chloroform, $[\alpha]_D^{25}$ + 165°, + 165°, + 166° (C 1.0) respectively; those of the white potato and sweet potato limit dextrins formed swollen, gelatinous masses that would not disperse. The white potato and sweet potato acetates contained higher percentages of phosphorus than the soluble acetates. Each limit dextrin acetate sintered in capillary tubes in the range 170°–190°C. and softened slowly thereafter up to 250°C.; however, the corn, wheat, and tapioca acetates became clear in the tubes at about 225°C., while the white potato and sweet potato acetates did not.

The end optical rotations of the triacetates of all the limit dextrins in the hydrogen bromide-acetic acid-acetyl bromide reagent of Jeanes and Hilbert (10) fell within the narrow range $[\alpha]_D^{25}$ + 15.8 ± 0.1° S. Since the experimental error was approximately 0.05° S, the different varieties of limit dextrins have the same rotation in the Jeanes-Hilbert reagent; hence, there is probably no great variation in the extent of branching among the different varieties of amylopectin.

The phosphorus contents of the amylopectins and limit dextrins are given in Table III. For the root and tuber starches the phosphorus contents of the limit dextrins are approximately double those of the amylopectins. This indicates that the phosphorus present in the potato, sweet potato, and tapioca starches is chemically bound in the half of the molecule remaining as limit dextrin. The small amount

of phosphorus in the corn and wheat amylopectins is not an integral part of the residual dextrin because the degradation of the cereal amylopectins resulted in a decrease in phosphorus content. These results are in accord with those of Posternak (21) and others (2, 22). Since

TABLE III

PHOSPHORUS CONTENTS OF AMYLOPECTINS AND LIMIT DEXTRINS

Source of amylopectin	Phosphorus		Ratio
	Amylopectin	Limit dextrin	Limit dextrin to amylopectin
	%	%	
Corn	0.009	0.005	0.6
Wheat	0.007	0.006	0.9
Sweet potato I	0.017	0.035	2.1
Sweet potato II	0.019	0.032	1.7
White potato I	0.093	0.182	2.0
White potato II	0.089	0.161	1.8
White potato IIa [1]	0.102	0.174	1.7
Tapioca	0.009	0.016	1.8

[1] Potato starch IIa was not treated with liquid ammonia before fractionation, otherwise the same as II.

Posternak showed white potato, arrowroot, and sago starches contain chemically bound phosphorus and the present work extends the list to include sweet potato and tapioca starches, it is probable that all root and stem starches contain bound phosphorus and only the cereal grains give starches with no significant amount of phosphorus in combination with the glucosidic units. The fact that the conversion limits of the corn and wheat amylopectins are slightly higher than those of the root and tuber amylopectins has been attributed to the presence of amylose impurity in the cereal amylopectins; on the other hand, the blocking of enzymic action at glucose units containing phosphorus would cause a similar result.

The iodine sorption values, expressed as milligrams iodine sorbed per gram of starch fraction, are listed in Table IV. Tapioca and white potato amylopectins and limit dextrins showed minimal sorptions of iodine. Corn and wheat amylopectins sorbed appreciable quantities of iodine, but in producing the limit dextrins much of the iodine-sorbing material was lost, probably because amylose was present as an impurity. The hydrolysis of sweet potato amylopectins to limit dextrins gave a different result. The two amylopectins sorbed about the same amount of iodine as corn amylopectin, but the limit dextrins retained more of the iodine-sorbing factor. A similar effect was obtained in corn amylopectin II that was isolated from the amylose fraction; the limit dextrin sorbed nearly as much iodine as the amylo-

pectin. This amylopectin may have contained a small amount of amylose as indicated by the higher alkali number in Table II; but the alkali number of the limit dextrin was lower and normal, showing that the alkali-labile impurity was removed by beta-amylase.

TABLE IV

IODINE SORPTIONS OF AMYLOPECTINS AND LIMIT DEXTRINS—CONCENTRATION 40 MG. PER 100 ML., TEMPERATURE 24°–26°C.

Source of amylopectin	Amylopectin	Limit dextrin
	mg./g.	*mg./g.*
Tapioca	3.0	1.9
White potato I	3.7	3.4
White potato II	4.2	3.4
Wheat	8.6	3.5
Corn I	15.3	7.7
Corn II [1]	18.1	16.7
Sweet potato I	16.0	12.4
Sweet potato II	16.3	11.8

[1] Corn amylopectin II was isolated from crude amylose-butanol precipitate by recrystallizing the amylose and recovering the solubles.

Since iodine-sorbing material is found in the limit dextrins after the completed action of beta-amylase, the presence of some molecules with a low degree of branching and /or relatively long, straight trunks is indicated. Two methods capable of removing linear amylose from branched amylopectin, adsorption on cotton and precipitation with *n*-butanol, were successful in removing a fraction (5–8%) from the limit dextrin of sweet potato I amylopectin. The butanol precipitate was not crystalline and sorbed much less iodine (36 mg./g.) than amylose (200 mg./g.), but much more than any amylopectin or limit dextrin (1–16 mg./g.). These phenomena are being investigated further to determine whether some amylopectins, especially those of sweet potato and corn, contain molecules intermediate in the extent of branching between amylose and the average for amylopectin.

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STUDIES ON EXPERIMENTAL BAKING TESTS. IV. COMBINED EFFECTS OF YEAST, SALT, AND SUGAR ON GASSING RATES [1]

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ABSTRACT

A baking formula of 3% yeast, 1.75% salt, 5% sugar plus an ammonium salt was found necessary for adequate gassing up to and through the pan-proof period under the A.A.C.C. standard procedure.

Increased sucrose in the formula augmented the initial gassing rate and diminished and delayed the fermentation attributed to maltose. Salt exhibited an over-all depressing and extending effect on fermentation. An interaction of salt and sugar in fermenting doughs was observed. The ammonium ion was the effective ion in the acceleration of maltose fermentation by ammonium dihydrogen phosphate.

Increasing yeast concentrations resulted in acceleration of fermentation and earlier exhaustion of fermentable sugars.

Baking data obtained with a formula based on these studies showed a very close relationship between protein content and loaf volume, i.e., gassing power had been eliminated as a variable and the loaf volume was strictly dependent on flour strength.

Gassing power and gas retention have long been considered independent variables influencing the baking properties of wheat flour doughs. Gassing power to a large degree is dependent on the formula used, while optimum gas retention is an inherent property of the flour itself, depending to great extent, though not entirely, on the protein content. Recent work has indicated that certain factors, formerly regarded as influencing gassing power alone, exert an effect on the gas

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retention. Sandstedt, Jolitz, and Blish (13), in a study on synthetic flours, demonstrated that removal of the amylodextrin, or small granule portion of the starch, produces improved loaf volume. Kneen and Sandstedt (5) observed that improvement results from addition of alpha-amylase preparations to sugar-rich doughs from which presumably gas production had been removed as a factor. But despite this evidence of an interaction of those flour components responsible for gas production and gas retention, the gassing power of a dough must still be largely governed by simple additions to the baking formula.

Larmour and Brockington (8, 9), and Larmour and Bergsteinsson (7), investigating the effects on gassing rates of various fermentation procedures, dosages of bromate, various acidic, basic, and neutral salts, and a number of the common sugars, concluded that the effects of bromate and of punching are negligible; that bakers' yeast distinctly prefers sucrose, glucose, and fructose to maltose; that apart from the influence of pH exerted by the acidic and basic salts, the presence of salt retards yeast fermentation. They observed also that the gassing-rate curves with small amounts of added sugar always showed double maxima, a phenomenon interpreted as due to preferential fermentation of sucrose by the yeast.

The yeast used by Landis and Frey (6) in a methods study produced a double maxima gassing-rate curve. Eisenberg (4), with three different commercial bakers' yeasts, obtained three different types of gassing curves with flour doughs, one type of which showed the first maximum reduced to a mere inflection.

Blish and Sandstedt (3) postulated the existence of "Factor M," a biocatalytic activator, specifically effective in the fermentation of maltose. Sandstedt and Blish (11, 12) presented confirmatory evidence for the existence of this factor and recommended proofing doughs to constant height in laboratory test baking in order to minimize the effects of differing contents of "Factor M."

Schultz, Atkin, and Frey (14) attributed the "Factor M" effect to lack of amino nitrogen, but Ofelt and Sandstedt (10) failed to equalize proofing rates with additions of amino nitrogen, although they did so successfully with 0.5% dosages of ammonium dihydrogen phosphate. Larmour and Bergsteinsson (7) had studied the effects of ammonium salts and had noted the specific effect of the ammonium ion on the rate of fermentation of maltose in flour doughs.

Atkin, Schultz, and Frey (1) developed a nutrient solution capable of supporting the fermentation of sucrose at a rate equivalent to that of a flour substrate. Ammonium compounds are not included among the many constituents of this medium, but amino nitrogen is required. Many of the constituents are present in flour, some of them

in excess amounts. There may be a parallelism here, the active components of the nutrient solution being not necessarily the active factors in a flour substrate.

Clarification of the exact role of those baking constituents that have a known effect on the gassing power of a dough may permit development of baking formulas where the gassing power is independent of flour sources. The baking test, under such formulas, would evaluate flour strength only, unobscured by other factors. The more common dough ingredients having a known effect on gas production are yeast, sugar, salt, and certain yeast foods.

This is the report of the results of a study of the action and interaction of these baking constituents on the gas production of doughs. Using the data obtained, the degree of relationship between protein content and baking data is determined.

Materials and Methods

Gas production and gas retention at 30°C. were measured in a volumetric apparatus similar to that of Bailey and Johnson (2). Dough aliquots corresponding to 25 g. of flour were used. Gas production was measured in burettes inverted over saturated salt solution and gas retention over 23% potassium hydroxide solution. Readings were taken at 10-minute intervals.

Although it is realized that a strict comparison to baking conditions ends at the time the loaf goes to the oven (3 hours and 55 minutes under standard A.A.C.C. procedure), measurements were taken beyond this point on the assumption that the behavior of the dough at extended periods at 30°C. may parallel its behavior in the oven.

The formula used for baking is as follows: 3% yeast, 5% sugar, 1.75% salt, 0.1% ammonium dihydrogen phosphate, 0.3% malt, 4% nonfat milk solids, 3% shortening, and 0.001% potassium bromate.

The flour series examined represented six subseries of 16 flours each, from individual varieties of hard red Canadian spring wheats. The protein range for the entire flour series was from 7.9 to 18.6% on 13.5% moisture basis.

Results and Discussion

Effect of Sucrose. The effects on fermentation rates of additions of sucrose to a simple saltless dough with 3% yeast are shown in Fig. 1-A. The yeast used for all this work gave sharply defined double-maxima rate curves, as can be seen in the curve for the sugarless dough (0%).

Added sucrose raises the first maximum, lowers the second, and delays its appearance. With increasing amounts of sucrose the min-

imum becomes less pronounced until with 5% dosage it disappears altogether, thus giving a curve with only one maximum. From this maximum the rate falls off fairly sharply at first and then more gradually, indicating that fermentation of maltose may be partially responsible for the gassing rate at later time intervals. Of course the 5%-sugar curve may be entirely the result of sucrose fermentation, to the exclusion of maltose, but this appears unlikely from scrutiny of the intermediate curves. In general, the effect of sucrose in delaying the effective period of, and diminishing the rate of, maltose fermentation is quite clear.

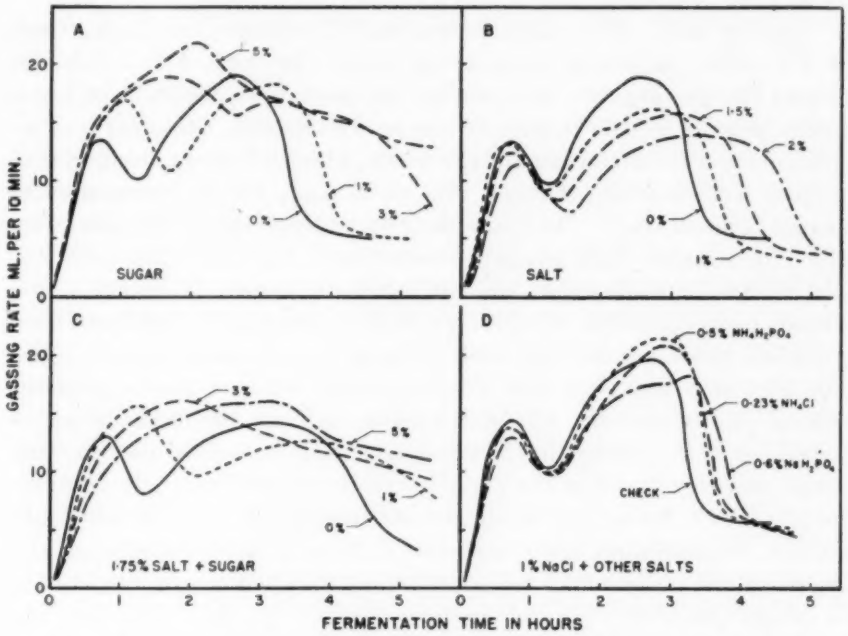

Fig. 1. Effects of various dough ingredients on gassing rates of doughs fermenting with 3% yeast. A. Sucrose dosages from 0 to 5%. B. Salt dosages from 0 to 2%. C. Sucrose dosages from 0 to 5% with 1.75% salt. D. Ammonium and phosphate salts with 1% sodium chloride.

The important thing to bear in mind with these rate curves is the total length of time over which a dough must gas in order to accomplish satisfactory rising during the pan-proof period. It is most essential to ensure good gassing between the third and fourth hours, the period during which the dough is proofing in the pan, because much of the conditioning effects of the dough during the mixing and fermentation period may be vitiated if in the last stage the gassing rate falls so low that the dough cannot become properly expanded. In Fig. 1-A both the sugarless and the 1%-sugar doughs exhibit a sharply declining rate of gassing during this critical period, and hence they

would not be adequately proofed by the end of the fourth hour and would therefore produce a small loaf of bread when proofed for a fixed time. On the other hand, both the 3%- and the 5%-sugar doughs maintain their gassing rates adequately during this period and hence could proof to the limit of their capacity to retain the gas.

While for purposes of conciseness and clarity, sugar dosages of 1, 3, and 5% only are shown in Fig. 1-A, intermediate values of 2 and 4% were also studied. These presented no anomaly and without graphical evidence it may be stated here that 2% sucrose is the minimum dosage capable of maintaining a relatively high gassing rate throughout the fourth hour.

Effect of Salt. The effects of varying salt dosages on gassing rates of 3%-yeast, sugarless doughs are shown in Fig. 1-B. Salt depresses the gassing rate but extends the period of relatively high gas production. Due to the relative size of the maxima, the effect is more noticeable on the latter part of the curve, which has been attributed to maltose fermentation, than on the initial part, which corresponds to sucrose fermentation. This has the effect of prolonging the time over which reasonably high gassing rates occur. For example, with 2% salt, without added sugar, the rate shows a broad maximum which extends from before the third hour to well beyond the fourth hour, thus providing stable continuous gassing during the pan-proof period. The rate, although less than with the lower salt dosages, averages about 13.5 ml. per 10 minutes, which, in a 100-g. formula, produces 300 ml. of carbon dioxide during the 55-minute period, sufficient to raise the dough properly if it has the capacity to retain the gas. By contrast the 1% curve shows that at the 3-hour time interval the gassing rate reaches its maximum and thereafter falls very sharply until at the fourth hour it is down to 5 ml. per 10 minutes, a rate wholly inadequate for proper proofing.

Maintenance of this adequate rate to the 4-hour point is accomplished by 1.75% (not plotted here) and 2.00% salt levels. These are the two levels most commonly used in commercial baking.

Combined Effects of Salt and Sugar. To determine the interaction of salt and sugar, both normally dough components under American baking practice, a study was made of the effects of sucrose at levels from 0 to 5%, on doughs having 1.50, 1.75, and 2.00% salt respectively. As the pattern of the gassing rate curves proved to be similar, only one is reproduced here, namely that at 1.75% salt with sugar at 0, 1, 3, and 5% levels. These curves are shown in Fig. 1-C.

It is interesting to note that while with 1% sugar the two maxima are clearly evident, at 3 and 5% sugar levels, the minimum has disappeared. Data not presented here show that this is true also for the

2% sugar level. As the two maxima are clearly evident with 3% sugar in a saltless dough (Fig. 1-A), and there is no indication of eradication of the minimum with any of the salt concentrations tested (Fig. 1-B), there must be a complementary effect due to the combined salt and sugar at this level of sugar.

These considerations together with other data too numerous to include here, Walden (15), indicate that the maximum gassing rate obtainable, with salt and sugar only, is an inverse function of the salt level. Increasing sugar merely delays attainment of this maximum set by the salt level and extends its duration.

Effects of Ammonium Salts. The stimulating effect of the ammonium ion on the rate of gassing is shown in Fig. 1-D. The curve marked "check" represents the rate with 3% yeast only. The data for the other three curves were obtained with doughs containing 1% sodium chloride, 3% yeast, no sugar, and amounts of ammonium chloride, ammonium dihydrogen phosphate, and sodium dihydrogen phosphate monohydrate respectively equivalent to 0.25% sodium chloride.

Despite the depressing effect of 1% sodium chloride (see Fig. 1-B), the small additional amount of the ammonium salts markedly increases the gassing rate at the second maximum. The two ammonium salts have almost identical effects in this respect. Sodium phosphate, on the other hand, depresses and extends the second maximum, behaving similarly to sodium chloride. It is thus evident that the effective ion is the ammonium ion, which confirms the conclusions of Larmour and Bergsteinsson (7).

Effect of Yeast Concentration. The combined effects of salt and sugar, in general, are to flatten out and extend the fermentation-rate curve, making it possible to achieve a well-sustained gassing rate through the pan-proof period, i.e., from the third to the fourth hour in the standard experimental baking procedure. The level of gassing will, however, depend on the amount of yeast present, and it is important, therefore, to choose a concentration that will ensure a sufficiently vigorous gassing rate to avoid the possibility of gassing becoming a limiting factor in the final loaf volume of the flour under test.

Two levels of yeast, i.e., 2 and 3%, were used with various sucrose dosages from 1 to 5%. Commercial and experimental practices correspond roughly to 2 and 5% sugar levels respectively, and these are the only values shown in Fig. 2. Other values being interjacent are omitted for purposes of simplification. The salt level of 1.75% was chosen as most nearly representative of baking practice and also one which extended the gassing rate to the 4-hour point.

The 3% yeast concentration starts the fermentation faster and the

rate goes higher in the initial phase than with the 2% concentration. After the first maximum has been passed at the end of 3 hours, three of these curves come close together and remain so until the 4.5 hour point when they diverge. These three curves are: 2% sugar, 3% yeast; 2% sugar, 2% yeast; and 5% sugar, 2% yeast. With the 2%-yeast level the yeast is the limiting factor in gas production during the 1.5 hour period from the 3-hour to the 4.5-hour points. With the 3%-yeast dough the high rate of fermentation achieved in the early phase exhausts the supply of added sucrose, and in the latter part of the period the limiting factor becomes the supply of maltose from the flour.

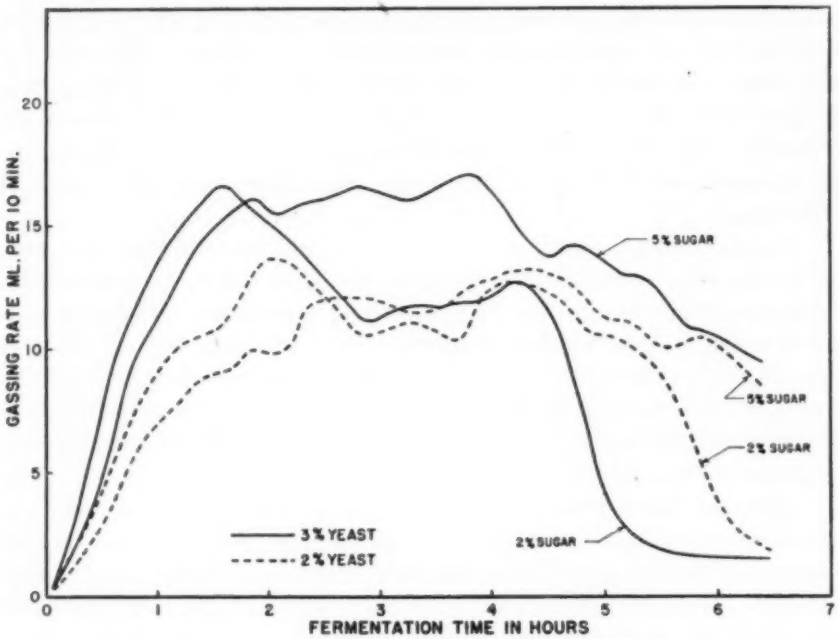

Fig. 2. Effects of sugar dosages on gassing rates of 1.75% salt doughs fermenting with 2 and 3% yeast.

With the 3%-yeast, 5%-sugar formula a high rate of gassing is maintained for 2 hours, i.e., between the second and fourth hours. With this formula it would be possible to ensure good gassing to the 5.5-hour point, because although by that time the rate has declined from the high of about 16 ml. per 10 minutes, it is falling slowly and is still at a higher value than the rates for the other three formulas during the critical period, i.e., between the third and fourth hours.

These data show that increased yeast tends to offset the depressing effects of increased salt; and that in order to ensure prolonged adequate gas production in doughs it is necessary to combine high salt, high

sugar, and high yeast concentrations. The levels of gassing shown here may not be necessary for commercial baking, but for experimental flour testing it is absolutely essential to provide conditions such that there are no factors likely to limit loaf volume other than the quality and strength of the flour itself. *The formula must ensure adequate gassing, especially throughout the pan-proof period, as otherwise the final volume may be merely a reflection of the failure of the formula rather than of the flour.*

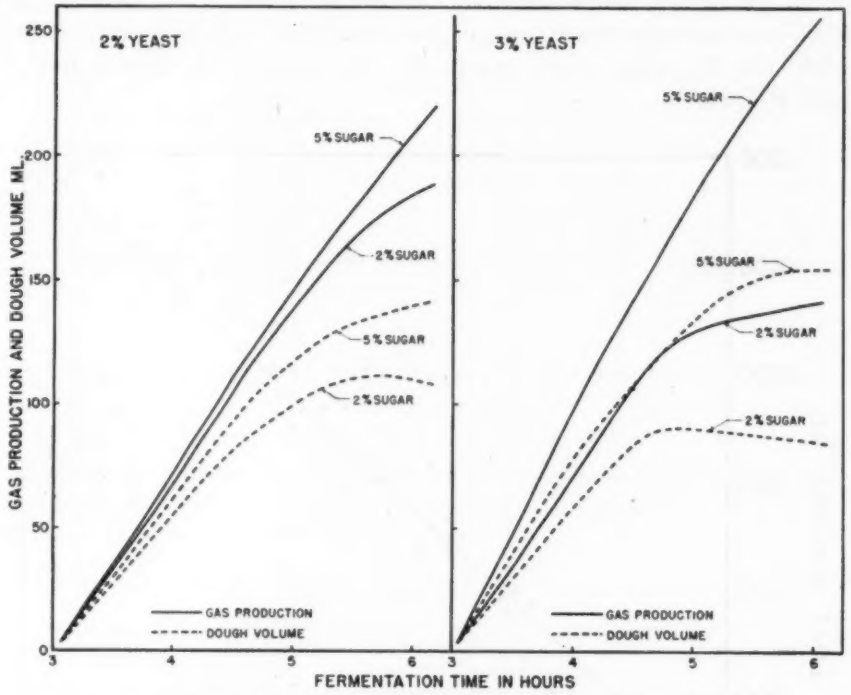

Fig. 3. Total gas production and dough volume changes of 1.75% salt doughs fermenting with 2 and 3% yeast at 2 and 5% sugar levels.

Dough Volume During Pan-proof Period. To test the foregoing conclusions further, the dough volumes and the total gas production of the doughs discussed in the preceding section were measured from the third hour to beyond the sixth hour. The results are shown graphically in Fig. 3.

Considering first the 2%-yeast doughs, at the end of the normal proofing period, i.e., at the fourth hour, the total gas produced by the 5%-sugar formula is 72 ml. and by the 2% sugar formula, 68 ml., the two being practically equal. The dough volumes are 60 ml. and 56 ml. respectively. At this 4-hour point, therefore, the amount of sucrose originally added has no appreciable differentiating effect on either the

total gas production or the volume of the dough. Hence the limiting factor for dough volume must be the yeast concentration.

With the 3%-yeast formula the total gas production with 5% sucrose at the 4-hour point is 100 ml., while for the 2% sucrose it is 70 ml., which is virtually the same value as for both sugar concentrations with the 2%-yeast formulas. Here, however, there is a wide difference between the two sugar concentrations, the 5% formula producing far more carbon dioxide than any of the other three formulas. The differences are further emphasized by the dough volumes, because in the 3%-yeast formulas the 5% sugar gives far higher volume at this point, while the 2% sugar gives practically the same as the two formulas with 2% yeast.

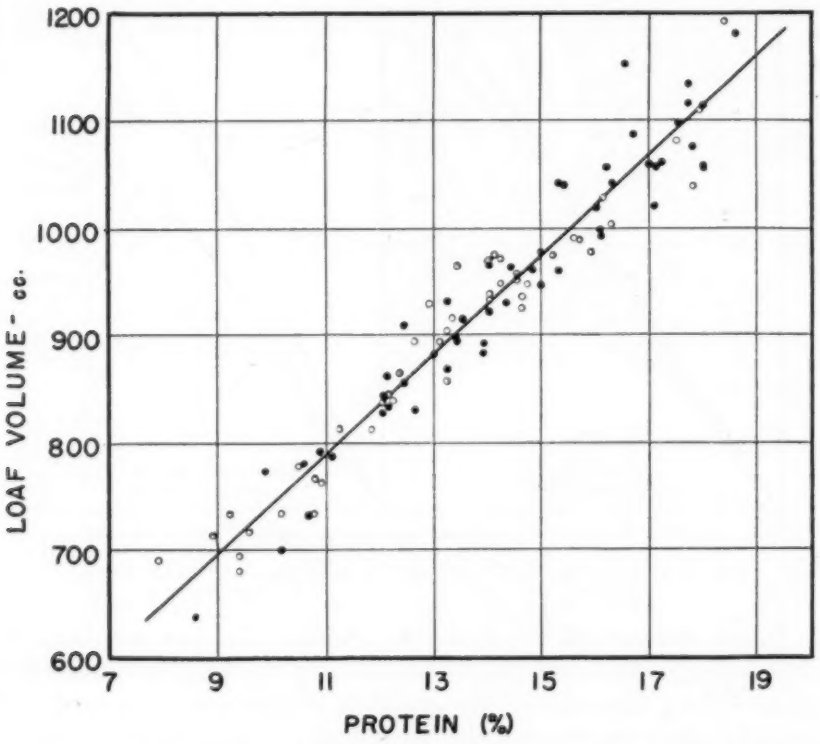

Fig. 4. Scatter diagram showing relation between loaf volume and protein content.

Further differences in the behavior of these four formulas are revealed by examination of the graphs beyond the 4-hour point. With 2% yeast the dough volumes at 5 hours are 118 and 100 ml. for the 5%- and the 2%-sugar formulas respectively, while with the 3% yeast they are 134 and 90 ml. for the 5%- and the 2%-sugar formulas re-

spectively. Here the 3%-yeast, 2%-sugar formula reflects the early exhaustion of the sugars, as shown in Fig. 2.

Thus, the high yeast, high salt, high sugar formula shown to be the most suitable on the basis of gassing power actually does increase the dough volume achieved during the pan-proof period as a result of its more adequate gassing rate.

Application to Baking. A series of 96 flours, baked by a high yeast, high salt, high sugar formula as described earlier showed exceedingly high correlation between loaf volume and protein content as evidenced by the data in Table I and Fig. 4.

TABLE I

STATISTICAL DATA FOR LOAF VOLUME AND PROTEIN CONTENT

	Entire series	Apex	Marquis	Rival	Renown	Regent	Thatcher
Mean protein of flour, %, $\bar{x}$	13.88	13.96	13.46	13.58	14.05	14.14	14.09
Mean loaf volume, cc, $\bar{y}$	923.4	905.2	908.5	901.3	930.1	935.3	959.8
Correlation coefficient, r_{xy}	+0.971	+0.991	+0.979	+0.982	+0.988	+0.994	+0.963
Regression coefficient, b_{yx}	+46.27	+37.08	+40.88	+52.64	+42.54	+46.04	+54.34
1% point	0.254	0.623	0.623	0.623	0.623	0.623	0.623

Although protein content is a strict measure of gluten quantity and not quality, this very close relationship indicates that there are few other factors concerned. It must be concluded, therefore, that the gluten quality of the flours was virtually the same throughout the series and that the gas production of the doughs was not a variable of any significance in relation to loaf volume.

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THE MOLD FLORA OF STORED WHEAT AND CORN AND ITS RELATION TO HEATING OF MOIST GRAIN[1]

CLYDE M. CHRISTENSEN and DOROTHY R. GORDON

ABSTRACT

All of more than 100 samples of corn, and several samples of wheat, collected from commercial lots were found to bear molds able to grow at relatively low moisture contents. The number of molds on corn increased with increasing moisture content of the seed. In both corn and wheat stored in vacuum bottles at different moisture contents, mold population and temperature increased with increasing moisture. At moisture contents favorable to their growth, the molds caused the temperature to rise to within a few degrees of the maximum the molds could endure. Two of the molds common on moist stored grain, *Aspergillus candidus* and *A. flavus*, grew on and heated moist sound wheat as rapidly, and to as high a temperature, as they did autoclaved wheat, while *A. glaucus* caused a higher rise in temperature of autoclaved than of sound wheat. Autoclaved moist wheat inoculated with 200,000 spores of *A. flavus* per gram heated to 45°C. in two days, while that inoculated with only 0.2 spore per gram heated to a comparable temperature in nine days, indicating that if conditions for mold growth are favorable, the amount of inoculum originally present may have only a minor effect on eventual heating. Under the conditions of the tests, none of the several fungicides used eliminated molds from moist, stored wheat, and thiourea appeared to be less toxic to *A. candidus* than to other common molds.

That molds may be responsible for heating and various deteriorative changes in moist stored seeds and other organic products has been shown by a number of workers (2, 3, 4, 6, 7, 8, 10, 13). Much of the work in this field up to 1944 has been summarized by Semeniuk and Gilman (12). Koehler (5) and Semeniuk, Nagel, and Gilman (13) have determined with considerable precision the moisture contents of

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seeds and relative humidities of air required for germination and growth of a number of mold species common on stored corn. The present work aimed to explore some of the major factors known, or presumed, to influence the growth of molds on stored corn and wheat, and thus to furnish a partial background of information for more intensive and critical studies.

Materials and Methods

In 1945, 100 samples of commercial corn, each weighing about four oz., were collected by Federal Grain inspectors at each of five terminals: Cedar Rapids, Iowa; Omaha, Nebraska; Minneapolis, Minnesota; Chicago, Illinois; and Toledo, Ohio. These were sent to the University Farm, St. Paul, in previously sterilized paper envelopes or bags. In 1946 a smaller number from various midwestern states were submitted by the Research Division of The Quaker Oats Co. Most of these were placed in one-pound tins as soon as collected, sealed, and shipped by air mail. The majority of them were received and assayed for molds the day after they were collected. No systematic collection of wheat was made, the lots tested having come from various sources as indicated in the presentation of results.

The mold assays were made by grinding the grain in a Wiley mill equipped with a 40-mesh sieve. The mill was cleaned between lots, and the first portion ground was discarded, a practice which preliminary tests showed would prevent any serious contamination from the preceding one. The resulting meal, collected in sterile paper bags, was cultured according to the technic described by Christensen (1). Several culture media were tested, including malt-salt, Czapek's, acid potato dextrose, Smith-Humfeld, and corn-meal agars; the malt-salt medium was found to be preferable, in that it generally yielded a larger number of a greater variety of molds than any of the others.

Water content of the grain was determined by drying two or three 25-g. portions to constant weight at 105°C. In comparative tests this was found to give results averaging within 0.1% of those obtained by the more precise oven-vacuum method. To determine the ability of various molds to heat grain, the seed, after any preliminary treatment as described under the individual experiments, was conditioned to the required moisture content, and 200-g. portions were placed in pint vacuum bottles; or the grain was placed in vacuum bottles, water added to bring it to the required moisture content, the bottles closed with rubber stoppers, placed in a horizontal position, and rotated and shaken occasionally during a 24-hour period, by which time it was assumed that the water was evenly distributed through the grain. The rubber

stoppers were then replaced with sterilized cotton plugs to permit air exchange.

This method is essentially the same as that used by some of the earlier workers (2, 3). It is, of course, less accurate than the methods more recently described (9, 11) in which adiabatic conditions are approached. The most serious objection to it is that the original moisture content of the material may be reduced by loss of vapor through the cotton plugs, or increased by the metabolic water from mold growth. Also, with time, the water tends to become unevenly distributed within the container. Recent work (8) indicates that it may be extremely difficult to maintain a uniform moisture content in grain on which molds are vigorously growing, even when the grain is ventilated with air of constant relative humidity. For these reasons, in the present study only approximate relationships between moisture content and mold growth could be determined.

Results

Mold Population of Commercial Samples of Corn and Wheat. The mold population of 120 samples of corn of the 1945 crop, and of 15 samples from the 1946 crop, is summarized in Tables I and II.

TABLE I

MOLDS PER GRAM IN CORN SAMPLES FROM FIVE MIDWEST TERMINALS, 1945 CROP

Grade	Maximum moisture content permissible, wet weight basis	Number of samples	Molds per gram	
			Range	Average
1	14.0	9	0–48,000	12,000
2	15.5	17	1450–522,000	96,000
3	17.5	21	0–1,470,000	262,000
4	20.0	16	5000–1,350,000	390,000
5	23.0	18	25,000–2,270,000	940,000
Sample		39	0–4,375,000	830,000

The species of molds were mainly those found by previous workers (4, 5, 13, 14) in corn and corn meal. In many samples typical "cob rotting" organisms were prevalent, such as *Fusarium moniliforme*, *Nigrospora sphaerica*, *Diplodia zeae*, *Cephalosporium acremonium*, and Penicillium spp. Koehler (5) showed that these are not able to grow in corn with a moisture content below 21 to 23%, and so ordinarily are of little significance in stored corn. In other samples *Aspergillus candidus*, *A. flavus*, *A. glaucus*, *A. niger*, *A. fumigatus*, and *A. versicolor* predominated. According to Thom and Raper (15) most of these are so-called "group" species, and each includes a number of different

TABLE II

MOLDS PER GRAM IN CORN FROM SEVEN MIDWEST TERMINALS, 1946 CROP

Source of sample	Moisture content	Molds per gram	Principal species of molds	Percentage of population
Akron, Ohio	16.0	8,000	*Monilia candida* *Aspergillus glaucus*	60 40
Akron, Ohio	17.3	110,000	*Fusarium moniliforme* *A. glaucus*	90 10
Cedar Rapids, Iowa	20.0	1,000,000	*M. candida*	95
Akron, Ohio	20.1	142,000	*F. moniliforme* *M. candida* *A. niger* and *A. flavus*	70 20 10
Akron, Ohio	20.2	1,600,000	*M. candida*	100
Lewisville, Ind.	20.5	20,000	Penicillium sp.	90
Chicago, Ill.	20.6	1,000,000	*M. candida*	100
Akron, Ohio	20.7	500,000	*M. candida*	99
Ames, Iowa	20.8	1,300	Penicillium sp. *A. niger* and *A. flavus*	50 50
Chicago, Ill.	21.5	1,800,000	*M. candida*	100
Sulfur Springs, Ind.	22.2	5,000	*M. candida* Penicillium sp. *A. niger* and *A. flavus*	30 40 30
Akron, Ohio	22.4	134,000	*M. candida*	95
Hallville, Ill.	22.9	96,000	Penicillium sp.	90
Cedar Rapids, Iowa	28.3	47,000	Mucor sp.	99
Cedar Rapids, Iowa	30.0	60,000	Mucor sp. *F. moniliforme*	85 15

types. In general, they are able to grow in materials whose water content is in equilibrium with an atmospheric relative humidity of 75 to 90%, and their occurrence in large numbers on many of these corn samples was only to be expected from the work cited above. Penicillium of undetermined species was found in nearly all of the samples, often being the dominant organism. Mucor spp. and Hormodendrum sp. predominated on a few of the lots, and were present on a majority of them.

The yeast-like organism, *Monilia candida* (apparently identical with *Candida albicans*), predominated in seven of the 15 1946 samples, growing on the outside of the seeds as an inconspicuous crust that on white corn was nearly invisible. Where present in amounts of several

hundred thousand per gram, it gave a decided sour and unpleasant yeasty odor to the corn. Pure cultures of the organism on various agar media have the same odor. Preliminary studies indicate that it will grow fairly rapidly on corn with moisture contents of 22 to 25%, but is not able to grow at moisture contents below 20%; also that it is a facultative anaerobe, able to grow slowly in moist corn stored in an atmosphere of carbon dioxide. It has since been found on various other lots of corn of the 1946 and 1947 crops.

The molds found on several samples of wheat are shown in Table III.

TABLE III

MOLDS PER GRAM IN SEVERAL SAMPLES OF WHEAT FROM VARIOUS SOURCES

Source	Crop year	Condition of wheat	Molds per gram	Kinds of molds
South Dakota	1945	Sound	2,040	Chiefly *Aspergillus glaucus*
South Dakota	1946	Sound	3,150	Chiefly *A. glaucus* and Penicillium sp.
Minnesota	1945	Sound	3,460	*A. glaucus* and *A. candidus*
Minnesota	1947	Immature	5,000	Penicillium sp., *A. glaucus*, Mucor sp., and Alternaria sp.
Southwestern U. S.	1946	Sound	2,600	*A. glaucus, A. candidus*
Southwestern U. S.	1946	Sick	66,000	*A. niger, A. flavus, A. candidus*, Penicillium sp., Mucor sp.

In general, a much less diverse flora was found on wheat than on corn, most probably because wheat normally is harvested with a much lower moisture content than corn. The principal organisms on all of the wheat samples tested were the species of *Aspergillus* adapted to low moisture contents, such as *A. glaucus*, *A. candidus*, and *A. ochraceus*, with Penicillium sp. and *A. flavus* being found occasionally in small numbers. The one sample of "sick" wheat tested had a much higher population of a more varied mold flora than the sound wheats.

Relation of Moisture Content and Mold Growth to the Heating of Corn. Corn of the 1945 crop which had been stored in a dry basement for nearly a year was moistened to six different water contents and 200 g. were placed in pint vacuum bottles, two bottles being used for each water content. The corn was not inoculated with molds, but bore a rather heavy and varied natural mold flora. The rise in temperature of this corn and the molds associated with it are shown in Table IV.

To determine the effect of some of the individual molds upon the heating of moist corn, spores from pure cultures were inoculated onto autoclaved corn which had been conditioned to various moisture con-

TABLE IV

HEATING OF NONINOCULATED CORN AT VARIOUS MOISTURE CONTENTS—NATURAL MOLD FLORA PRESENT

Water, per cent, wet weight basis	Temperature, °C.			Molds present at end of test
	4 Days	8 Days	12 Days	
16	24	25	24	*Aspergillus glaucus*, sparse conidia
18	25	26	26	*A. glaucus*, perithecia abundant
20	26	27	29	Mainly *A. glaucus*, some *A. candidus*
22	35	47	50	Mucor, *A. flavus*, *A. candida*,
24	33	45	50	*A. terreus*, *A. flavus*
26	45	51	53	*A. fumigatus*

tents with hot sterile water after the autoclaved corn had been placed in sterile vacuum bottles. The temperature of this corn dropped to that of the room in approximately 24 hours. The results are presented in Tables V and VI.

TABLE V

INFLUENCE OF THREE DIFFERENT MOLDS ISOLATED FROM HEATING CORN UPON THE HEATING OF AUTOCLAVED CORN AT THREE DIFFERENT MOISTURE CONTENTS

Mold	Days after inoculation	Moisture content, per cent of wet weight		
		18–19	20–21	22–23
Aspergillus candidus	4	40 [1]	42	45
	20	44	44	45
A. flavus	4	46	43	—
	20	45	43	—
Penicillium sp.	4	29	29	28
	20	34	34	35

[1] Temperature, °C.—room temperature 22°–25°C.

TABLE VI

HEATING OF AUTOCLAVED CORN AT DIFFERENT MOISTURE CONTENTS, INOCULATED WITH DIFFERENT MOLDS ISOLATED FROM HEATING CORN

Mold	Water, per cent of wet weight				
	14	16	18	20	22
A. candidus	20 [1]	21	26	43	40
A. flavus	22	22	22	41	—
A. terreus	20	20	21	34	44
Control (not inoc.)	21	—	21	—	21

[1] Temperature after 12 days, °C.

Supplementary tests, in which these molds were grown in pure culture on nutrient agar in petri dishes exposed continuously to various temperatures, indicate that the maximum temperature induced by each mold on autoclaved corn in vacuum bottles was within 2°–3°C. of the maximum temperature at which the organism concerned could grow.

Relation of Moisture Cortent and Mold Growth to the Heating of Wheat. Two varieties of wheat—Marquis, grown in Montana and nine years old at the time of the tests, germinating 90% (a number of seeds germinated slowly, indicating a decreasing viability), and Rival, from Minnesota, one year old and germinating 95%, with normal vigor—were conditioned to moisture contents of 20.0, 22.5, and 25.0%, 200 g. of each variety at each moisture content, placed in each of two vacuum bottles, and stored in the laboratory. After 11 days' storage, the temperatures in the bottles were recorded, the seed in each bottle was poured into a sterile paper bag held closely around the neck of the

TABLE VII

RELATION OF WATER CONTENT TO MOLD INCREASE AND TEMPERATURE RISE IN TWO VARIETIES OF WHEAT STORED IN VACUUM BOTTLES 11 DAYS—ROOM TEMPERATURE 22°–23°C.

Wheat	Water, per cent of wet weight	Temperature, °C.	Molds per gram	Species	Per cent
Marquis	20.0	25.0	720,000	*A. glaucus*	90
				Penicillium	5
				A. candidus	5
Rival	20.0	23.5	880,000	*A. glaucus*	80
				Penicillium	10
				A. candidus	5
				Others	5
Marquis	22.5	25.0	2,235,000	*A. glaucus*	50
				Penicillium	40
				A. candidus and *A. niger*	10
Rival	22.5	25.0	1,623,000	Penicillium	40
				A. candidus	30
				A. flavus	20
				A. glaucus	10
Marquis	25.0	39.0	173,000,000	*A. flavus*	80
				A. candidus	10
				Penicillium	5
				A. glaucus and *A. niger*	5
Rival	25.0	29.0	5,150,000	*A. flavus*	80
				Penicillium	15
				A. niger	5

bottle to prevent undue escape of spores, and mixed by shaking the bag. Several grams of this seed were removed from the bag, dried at room temperature in a sterile dish, then ground and the mold population determined. The results are given in Table VII.

An approximately similar mold flora developed on the two varieties, and the number of molds per gram were roughly proportional to the rise in temperature. Marquis heated more rapidly than Rival, and a larger population of molds developed on it, presumably because of its greater age, lower vitality, and thus greater susceptibility to invasion by molds.

To compare the rate of temperature increase induced by certain of the molds on living wheat with that on autoclaved wheat, a Montana hybrid wheat almost free of internal mold infection, germinating 95%, was surface disinfected by using a 0.5% solution of sodium hypochlorite as conditioning water. Previous tests on this seed lot had indicated that this would free it almost completely of molds, although this has not been true of other seed lots so tested. Rival wheat, also germinating 95%, was autoclaved at 15 lbs. pressure for 30 minutes, placed in sterile vacuum bottles, and conditioned with hot sterile water. After the water was presumed to be evenly distributed, by which time the temperature of the seed had dropped to that of the room, the two different grains were inoculated with three different molds. The results are shown in Tables VIII and IX.

TABLE VIII

HEATING OF AUTOCLAVED RIVAL WHEAT WITH DIFFERENT MOLDS—MOISTURE CONTENT 30% OF WET WEIGHT

	Temperature, °C., in duplicate bottles		
	5 Days	7 Days	8 Days
Aspergillus glaucus	26, 27	31, 34	38, 38
A. candidus	26, 32	43, 45	47, 47
A. flavus	43, 40	47, 47	45, 44

TABLE IX

HEATING OF SURFACE-DISINFECTED MONTANA WHEAT INOCULATED WITH DIFFERENT MOLDS—MOISTURE CONTENT 30% OF WET WEIGHT

	Temperature, °C., in duplicate bottles			
	5 Days	7 Days	8 Days	14 Days
Aspergillus glaucus	27,* 28	26, 27	25, 27	28, 31
A. candidus	44, 45	42, 43	44, 44	45, 46
A. flavus	45, 45	44, 45	44, 44	45, 46

Aspergillus glaucus heated the autoclaved grain faster, and to a higher final temperature, than it did the nonautoclaved grain. *A. candidus* and *A. flavus*, however, grew on and heated the nonautoclaved, living seed just as rapidly as they did the autoclaved seed. This is in agreement with the results of Milner, Christensen, and Geddes (6) in which it was shown that *A. candidus* and *A. flavus* rapidly reduced the viability of wheat at 18% moisture, being fairly vigorous facultative parasites of such dormant seed, while *A. glaucus* reduced the viability much more slowly. This test suggests that seed "condition" may determine to some extent the growth of certain molds and thus may be an important factor in the storage of grain.

Relation of Amount of Inoculum to Rate of Mold Increase and Temperature Rise of Moist Wheat. Rival wheat was autoclaved at 15 lbs. pressure for 30 minutes, and 200-g. lots were placed in sterile pint vacuum bottles. Inoculum was prepared by suspending spores of *Aspergillus flavus*, isolated from heating wheat, in sterile water to which one part of Vatsol (a wetting agent) to 10,000 parts of water had been added before sterilizing, to reduce the surface tension and facilitate a uniform suspension of spores. The original suspension was diluted 1:100, 1:10,000, and 1:1,000,000. The 1:10,000 dilution was cultured, and yielded 100 colonies per milliliter. Microscopic examination of the culture dishes after two to three days showed that over 90% of the colonies developed from single spores. A sufficient

TABLE X

INFLUENCE OF DIFFERENT AMOUNTS OF INOCULUM OF *Aspergillus flavus* ON THE HEATING OF AUTOCLAVED CORN CONTAINING 25% MOISTURE, WET WEIGHT BASIS

Number of spores of *A. flavus* per gram of corn	Temperature, °C., average of two bottles								
	Days								
	2	3	4	5	6	7	8	9	10
200,000	45	46	47	47	—	—	—	—	—
2,000	30.5	39	46	46	—	—	—	—	—
20	26	28	41	46	—	—	—	—	—
0.2	25	25	26	26	28	33	40.5	46	—
0.0 (Control)	24	24	24	23	23	23	26.5	30	38.5 [1]

[1] Heavily contaminated with *A. flavus*.

amount of the spore suspension of each dilution was added to the grain in each of two vacuum bottles to attain the desired moisture content. The bottles were then plugged with sterile rubber stoppers and occasionally rotated and shaken for 24 hours, after which the rubber stoppers

TABLE XI

EFFECT OF VARIOUS MOLD INHIBITORS UPON MOLD DEVELOPMENT IN, AND HEATING OF, MOIST WHEAT

Moisture content of grain, per cent of wet weight	Inhibitor	Concentration	When added	Effect on temperature	Condition of seed at end of test
25	Chloramine B	1:100	After temperature of grain had risen to 31°C.	Reduced temperature of grain to that of room (27°C.) for 5 days; temperature then rose again and in 10 days reached 42°C.	Heavily molded, Mucor sp. especially prevalent
30	Chloramine B	1:100	When moist grain was placed in vacuum bottle	Temperature rose slowly to 31°C.	Seed at bottom of flask bound together with mold
25	Spergon dust	1:1000	After conditioned grain was inoculated with *Aspergillus flavus*	Temperature rose to 45°C. in 14 days, only slightly slower rise than in controls	Heavily molded
25	P-toluensulfonilamide	1:1000	After conditioned grain was inoculated with *Aspergillus flavus*	Temperature rose to 45°C. in 14 days, only slightly slower rise than in controls	Heavily molded
25	Chloramine B	1:1000	After conditioned grain was inoculated with *Aspergillus flavus*	Temperature rose to 45°C. in 14 days, only slightly slower rise than in controls	Heavily molded
25	Thiourea	1:500	When conditioned grain was placed in bottles; grain not inoculated with molds	Temperature rose to 36°C. in 18 days; controls rose to 46°C. in 10 days	Heavily molded with *Aspergillus candidus*

were replaced with sterile cotton plugs. Sterile water was added to the two controls. The temperature rise as related to spore load is given in Table X.

The controls remained at room temperature for eight days, at which time one began to heat, followed shortly after by the other. Both proved to be contaminated with *Aspergillus flavus*. In most of these experiments with moist grain in vacuum bottles it has proved difficult to keep molds from entering the control bottles, especially if thermometers are inserted through the cotton plugs at intervals to ascertain the temperature. In all bottles in this test, by the time the temperature had increased to the maximum, the seed was almost obscured by the spores of *A. flavus*.

Effect of Various Inhibitors on the Development of Molds and the Heating of Moist Wheat. To separate the deterioration of stored seeds induced by molds from that due to chemical processes in the seeds, themselves, it is necessary to obtain seed free of molds. The present test was not intended to measure the relative fungicidal efficiency of the compounds used, but rather to determine whether any of them might eliminate molds from grain stored under conditions near the optimum for mold growth. In some cases the fungicide was dusted on the wheat after it had been conditioned; in other cases it was added to moist seed which had already begun to heat. The results of these tests are presented in Table XI.

It is obvious that none of these compounds eliminated molds from the seed. Thiourea, considered by Milner, Christensen, and Geddes (8) to be the most effective moldicide of the compounds they tested, in the present test permitted a vigorous growth and sporulation of *Aspergillus candidus*, but apparently inhibited other molds almost completely. That a given compound may be effective against certain organisms but not others, or effective under certain conditions but not others, is too well known to need emphasis, and perhaps the chief value of the above tests is to indicate that this elementary concept must be kept in mind in work with stored seeds.

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FACTORS AFFECTING THE DETERMINATION OF THIAMINE AND RIBOFLAVIN IN ENRICHMENT PREMIXES CONTAINING FERRUM REDUCTUM [1]

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ABSTRACT

Acid extraction of flour premixes containing ferrum reductum causes much greater losses of thiamine and riboflavin than are found when acid solutions of the vitamins are shaken with ferrum reductum under identical conditions. This difference arises from the fact that the vitamin losses occur chiefly during the interval in which the vitamins are going into solution. The effects of pH, specific buffer, and time of extraction on such vitamin losses from flour premixes are similar to those previously reported in model experiments with vitamin solutions wherein higher ratios of iron to vitamin were employed.

Increasing the acid concentration in the extraction of a flour premix containing ferrum reductum decreases the thiamine and riboflavin losses. If such premixes are extracted at 100°C. with a 0.1 *N* acid solution of cystine, at least 1.5 mols of cystine must be used per mol of iron to avoid vitamin losses.

Corn grits premixes containing ferrum reductum and vitamins in a limestone base also suffer high losses of thiamine and riboflavin during acid extraction. The high ratio of limestone to vitamins has little effect on the vitamin losses, in contrast to the marked reduction in these losses when similar quantities of flour or starch are mixed with a flour premix sample prior to hot acid extraction.

Thiamine and riboflavin are partially destroyed when flour enrichment premixes or other products containing large amounts of ferrum reductum are assayed by extraction in the usual acid extractants (3). No such losses occur when the iron is present as ferrous or ferric salts. The means devised to circumvent this effect in the presence of ferrum reductum include extraction at pH 6–6.5, where little or no iron goes into solution, and extraction with acid containing cystine, which is apparently reduced preferentially in place of thiamine or riboflavin. Other evidence has been adduced which indicates that the effect on these vitamins is reductive.

It has been noted in the course of this work that the *magnitude* of the vitamin losses from flour premixes is considerably greater than from model solutions of comparable concentration containing only the vitamins and iron. The present report details experiments bearing on this point as well as data on the effect of ferrum reductum in corn meal premixes and in enriched flour.

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Materials and Methods

Materials. The flour enrichment premixes were "double-strength" commercial premixes from three different manufacturers, containing, according to label claim, the following amounts of vitamins and iron, expressed in mg. per oz., in a starch base: thiamine, 760; riboflavin, 460; niacin, 5480; iron, 4800.

The corn meal premixes were laboratory batches containing limestone as a source of calcium and as a carrier for the vitamins and iron. Other laboratory-prepared premixes, as well as solutions, were made with U.S.P. crystalline vitamins and U.S.P. ferrum reductum, unless otherwise specified.

Analytical Methods. Thiamine was determined in premixes in direct dilutions, i.e., without the Decalso step, by the thiochrome method of Hennessey (4), which gave results equal to or slightly higher than colorimetric assays by the procedure of Hochberg, Melnick, and Oser (5). The latter is affected in some cases by inhibitors which can be removed by Decalso treatment (3).

Riboflavin was determined by direct fluorometric measurement with a hydrosulfite blank (3), and iron was determined colorimetrically by the A.A.C.C. α,α'dipyridyl method (1).

Experimental Results

Comparison of Solid Vitamins and Solutions. In studies of the mechanism of the destruction of thiamine and riboflavin by acid extraction in the presence of ferrum reductum, it was found that shaking an acid *solution* of thiamine and riboflavin with ferrum reductum—with all components at the same concentrations as in the acid extraction of a flour premix—caused much smaller vitamin losses than were found in the case of flour premixes (cf. Table I). Since it appeared possible that the ingredients of the premixes might be enhancing the destructive effect of the ferrum reductum, the above experiment was repeated with C.P. ferrum reductum and also with starch and starch plus niacin added with ferrum reductum to the vitamin solution in premix proportions before shaking. Rice, wheat, and corn starches were also compared, but in no case was the vitamin recovery decreased by addition of one or a combination of these ingredients. Hence, the quantitative difference in the effect of ferrum reductum plus acid on premixes and vitamin solutions appears to lie in the fact that the vitamins are present in the one case as solids and in the other case in solution. To test this point further, dry mixtures were prepared with the same ingredients as in the above experiments, ranging from complete premixes to mixtures of a single vitamin and ferrum reduc-

tum. After shaking under the same conditions, thiamine and riboflavin losses were in all cases of the same order of magnitude as for the commercial premix and much greater than for comparable vitamin solutions.

TABLE I

DESTRUCTION OF THIAMINE AND RIBOFLAVIN BY FERRUM REDUCTUM DURING 30-MINUTE EXTRACTION IN 0.1 *N* SULFURIC ACID SOLUTION AT 24°C.—COMPARISON OF CRYSTALLINE VITAMINS AND VITAMIN SOLUTIONS

Method of adding vitamins	Content of test sample					Vitamin recovery	
	Thiamine	Riboflavin	Niacin	Ferrum reductum	Starch	Thiamine	Riboflavin
	mg.	*mg.*	*mg.*	*mg.*	*mg.*	%	%
0.150 g. commercial flour premix	4.0	2.6	28.6	27.8	87 (Wheat)	47	35
Vitamin solution	4.0	2.6	0	28 (USP)	0	92	93
Vitamin solution	4.0	2.6	0	28 (CP)	0	91	96
Vitamin solution	4.0	2.6	0	28	85 (Corn)	94	96
Vitamin solution	4.0	2.6	29	28	85 (Corn)	91	97
Vitamin solution	4.0	2.6	29	28	85 (Rice)	94	96
Vitamin solution	4.0	2.6	29	28	85 (Wheat)	93	99
Crystalline vitamins [1]	4.0	2.6	29	28	85 (Wheat)	45	42
Crystalline vitamins	4.0	2.6	29	28	0	41	42
Crystalline vitamins	4.0	2.6	0	28	0	46	43
Crystalline vitamins	4.0	0	0	28	0	40	—
Crystalline vitamins	0	2.6	0	28	0	—	39

[1] Mixtures were prepared by shaking the dry ingredients in the flask before extraction.

Effect of pH. In view of the unusual effects of pH and buffer ions on the action of ferrum reductum on solutions of thiamine and riboflavin reported by De Ritter and Rubin (3), and the differences between solid vitamins and solutions given in Table I, a study was made of the effect of pH in these buffers on vitamin losses from flour premix. Acetate buffer was studied over the range 3.0 to 6.5 and McIlvaine's phosphate-citric acid buffer series from pH 2.2 to 8.0 (2). In each series, 0.150 g. of premix was shaken 30 minutes at 24°C. with 300 ml. of buffer solution. The acetate buffer series was prepared by titrating 20, 30, 40, 50, and 60 ml. of 2.5 *M* sodium acetate with 5 *N* sulfuric acid solution to pH 3, 4, 5, 6, and 6.5, respectively, before dilution to 300 ml. For the phosphate-citrate buffer, 100 ml. of mixed buffer solutions at the desired pH were diluted to 300 ml. After shaking the flour premix with these solutions, diluting to 500 ml. and filtering, the pH of each extract was measured. The pH in each case was within 0.1 of the original value except for the lowest pH in each series which increased from 3.0 to 3.4 in the acetate buffer and from 2.2 to 2.4 in the phosphate-citrate buffer. Thiamine, riboflavin, and iron were

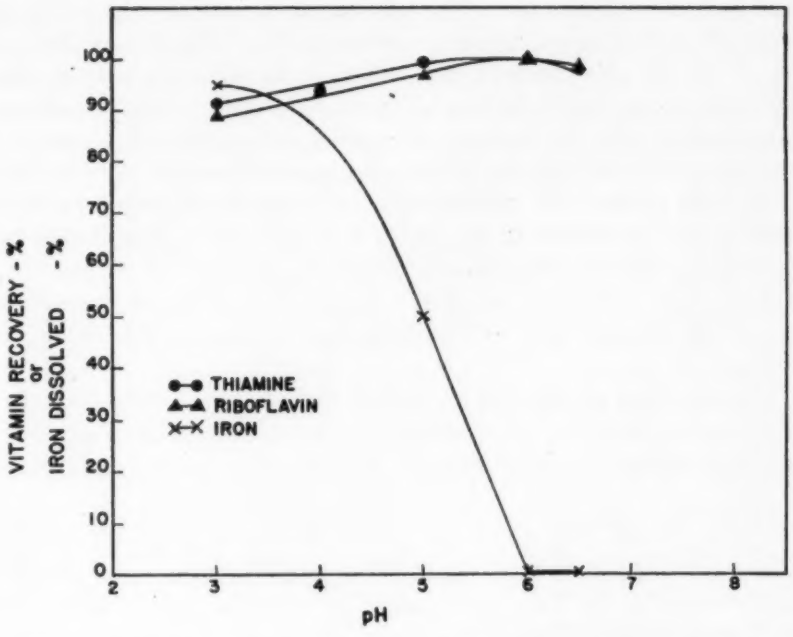

Fig. 1. Effect of pH on (*a*) recovery of thiamine and riboflavin and (*b*) solubility of iron in extraction of flour premixes containing ferrum reductum with acetate buffer.

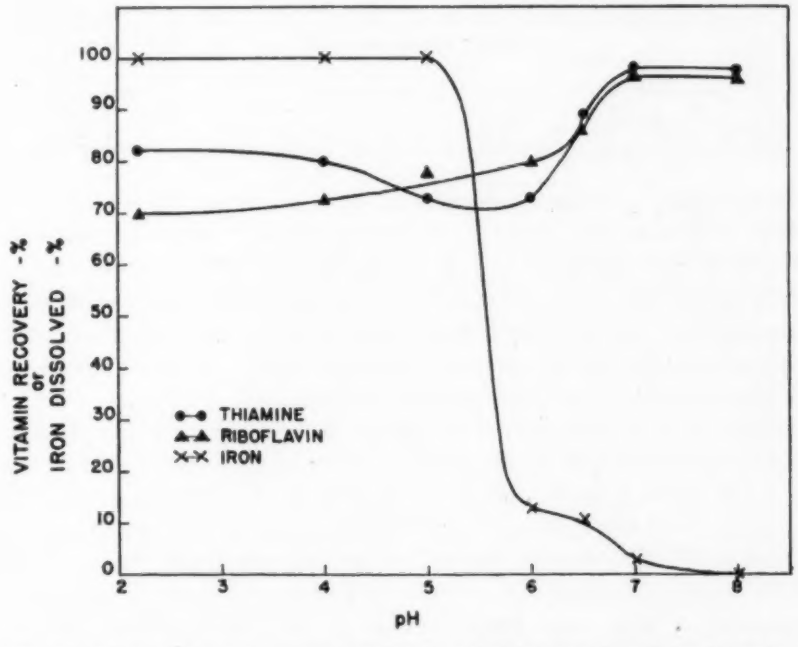

Fig. 2. Effect of pH on (*a*) recovery of thiamine and riboflavin and (*b*) solubility of iron in extraction of flour premixes containing ferrum reductum with phosphate-citrate buffer.

determined in each extract. The vitamin recovery and the extent of solution of the ferrum reductum are shown in Fig. 1 for the acetate and in Fig. 2 for the phosphate-citrate buffers. At lower pH values, vitamin recoveries are much higher in the acetate than in the phosphate-citrate buffer. In the former, recoveries are practically complete above pH 5, whereas in the latter, complete recoveries are obtained only at a pH of about 7. The ferrum reductum in the premix is more soluble in the phosphate buffer, but there is no direct correlation between the amount of iron dissolved and the amount of vitamins destroyed. In both buffers, there is a sharp increase over a narrow pH range in the amount of iron dissolved, but no appreciable change in the vitamin recovery.

Effect of Time of Shaking in 0.1 N Sulfuric Acid Solution. The *rate* of loss of thiamine and riboflavin in extraction of flour premixes with 0.1 *N* sulfuric acid solution at 24°C. was determined by shaking

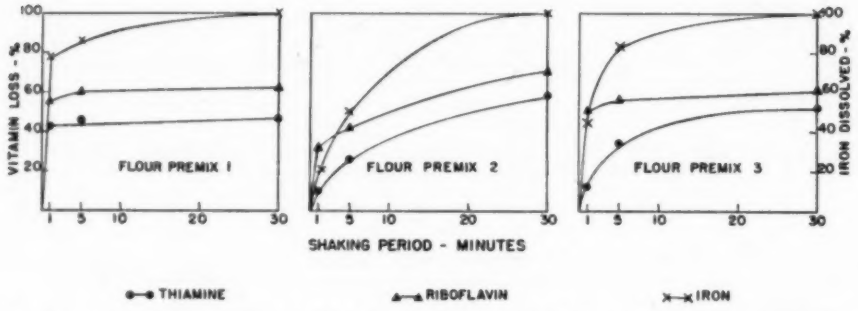

Fig. 3. Effect of time of shaking in 0.1 *N* sulfuric acid solution at 24°C. on (*a*) loss of thiamine and riboflavin and (*b*) extent of solution of iron from flour premixes containing ferrum reductum.

0.150 g. samples of three commercial premixes with 300 ml. of 0.1 *N* sulfuric acid solution for 1, 5, and 30 minutes. Vitamin losses and amounts of iron dissolved are shown in Fig. 3. It is evident that the destruction of both vitamins and the solution of the ferrum reductum occur rapidly. In this case, there appears to be a definite correlation between vitamin losses and iron solubility since all three curves for each premix follow the same general pattern.

Effect of Acid Concentration. Since it appears that the vitamin losses occur chiefly during the interval in which the vitamins are going into solution, it was of interest to determine whether increasing the concentration of the acid extractant would increase the rates of solution and consequently decrease the thiamine and riboflavin losses. Thus 0.150 mg. of a flour premix containing ferrum reductum were shaken 30 minutes at 24°C. with 100 ml. of acid. The effect of the acid concentration on the vitamin losses is shown in Table II. Both thiamine

and riboflavin show progressively higher recoveries with increasing acid concentration, the rate of increase in recovery being somewhat greater in the case of thiamine.

TABLE II

EFFECT OF ACID CONCENTRATION ON VITAMIN LOSSES FROM FLOUR PREMIX
(Extraction with sulfuric acid solution at 24°C.)

Acid normality	Vitamin recovery	
	Thiamine	Riboflavin
	%	%
0.1	44	40
0.2	51	44
0.3	56	45
0.5	64	53
1.0	73	60
2.0	83	68

Minimum Amount of Cystine Required. Since a large excess of cystine in acid solution has been shown (3) to protect thiamine and riboflavin in flour premixes from destruction by acid plus ferrum reductum, experiments were performed to determine the minimum cystine/iron ratio required for complete protection. Thus 0.150 g. of flour premix was extracted 30 minutes at 100°C. with 300 ml. of 0.1 *N* sulfuric acid solution containing graded amounts of cystine as given in Table III. Thiamine and riboflavin assays of filtered extracts indicate that 1.5 mols of cystine per mol of iron is sufficient in all cases.

TABLE III

MINIMUM AMOUNT OF CYSTINE REQUIRED FOR HOT ACID EXTRACTION OF FLOUR PREMIXES CONTAINING FERRUM REDUCTUM

Mol cystine per mol iron	Vitamin recovery			
	Thiamine		Riboflavin	
	Premix 1	Premix 2	Premix 1	Premix 2
	%	%	%	%
0	88	71	76	68
0.15	99	87	91	98
0.30	100	96	92	97
0.75	99	95	96	100
1.5	100	98	100	99
3.0	99	98	98	100

Stoichiometrically 1 mol of cystine is required in the oxidation of 1 mol of Fe° to Fe^{++}. However, an excess of cystine up to 3 mols per mol of iron does not interfere.

Extraction of Corn Meal Premixes. The effect of acid extraction on another product containing a relatively large amount of iron is

illustrated by the data in Table IV on corn grits premixes. Because of the high concentration of limestone in these premixes, it was necessary to use 500 ml. of 0.2 *N* sulfuric acid solution per gram of premix to keep the pH down to a level (approx. 1.0) at which no thermal destruction of vitamins would occur at 100°C. Extraction with an acid solution of cystine at 100°C. and with pH 6.0 acetate buffer, both of which are safe in the presence of ferrum reductum, was used as a basis of comparison. The premixes containing ferrum reductum suffered thiamine losses of 19 and 30% and riboflavin losses of 35 and 38%, respectively, during hot acid extraction. No vitamin loss was caused by acid extraction of the premixes containing sodium iron pyrophosphate.

TABLE IV

COMPARISON OF EXTRACTION PROCEDURES FOR CORN MEAL PREMIXES

Corn meal premix no.	Type of iron	Extractant		
		0.2 *N* H_2SO_4 at 100°C.	0.2 *N* H_2SO_4 + cystine at 100°C.[1]	pH 6 acetate buffer at 24°C.
		THIAMINE CONTENT—mg./oz.		
1	Ferrum reductum	3.9	4.7	4.9
2	Ferrum reductum	7.9	11.5	11.1
3	Sodium iron pyrophosphate	5.4	5.2	5.2
4	Sodium iron pyrophosphate	11.3	11.7	11.3
		RIBOFLAVIN CONTENT—mg./oz.		
1	Ferrum reductum	11.7	18.0	18.1
2	Ferrum reductum	10.0	16.0	16.0
3	Sodium iron pyrophosphate	18.1	18.2	18.1
4	Sodium iron pyrophosphate	16.0	15.9	16.0

[1] 3 mols of cystine in acid solution per mol of ferrum reductum.

Protective Effect of Flour. Acid extraction of enriched 80% extraction flour containing ferrum reductum has been shown by De Ritter and Rubin (3) to entail no loss of either thiamine or riboflavin. To determine whether this lack of effect is due to the protective effect of the flour itself, 0.150 g. samples of three flour premixes containing ferrum reductum were mixed with 10 g. of such a flour before extraction with 300 ml. of 0.1 *N* sulfuric acid solution at 100°C. Comparative results for these premixes with and without added flour are given in Table V. The addition of flour provides almost complete protection

to both thiamine and riboflavin which, in the absence of flour, are destroyed to the extent of 15–25% and 32–40%, respectively, under the same conditions.

TABLE V

PROTECTIVE EFFECT OF FLOUR DURING EXTRACTION OF FLOUR PREMIXES
(In 0.1 *N* sulfuric acid solution at 100°C.)

Flour premix no.	Vitamin recovery			
	Premix only		Premix + flour [1]	
	B_1	B_2	B_1	B_2
	%	%	%	%
1	85	68	97	96
2	77	66	97	98
3	75	60	98	98

[1] 10 g. of 80% extraction flour mixed with 0.150 g. of flour premix before extraction.

In a similar experiment in which 10 g. of wheat starch were used in place of the 10 g. of flour, the starch was found to be almost as effective as the flour in preventing the vitamin losses. Hence, it appears that this protective effect of flour is due to the fine, starchy particles and is probably a physical rather than a chemical action.

Discussion

Acid extraction of flour enrichment premixes containing ferrum reductum causes much greater destruction of thiamine and riboflavin than is found on shaking acid solutions of the vitamins with ferrum reductum at comparable concentrations. This difference cannot be attributed to any of the other premix ingredients, but arises solely from the fact that the vitamins are present in one case as solids and in the other case in solution. Hence, it appears that the vitamin losses occur chiefly during the period in which the vitamins are going into solution. The high initial rates of loss shown in Fig. 3 confirm this observation.

The effect of pH in acetate buffer over the range 3 to 6.5, and in phosphate-citrate buffer from 2.2 to 8, is similar for the flour premix to that observed by De Ritter and Rubin (3) for vitamin solutions shaken with a much greater excess of ferrum reductum. Vitamin losses from the premix are small in acetate buffer and practically negligible above pH 5.0. In phosphate-citrate buffer, vitamin recoveries are poor up to pH 6.5 and only approach 100% from pH 7 to 8. The extent of these losses at various pH levels cannot be correlated with the amount of ferrum reductum dissolved. In 0.1 *N* acid extraction of flour premixes at 24°C., however, the rate of loss of both vitamins appears to be directly related to the rate of solution of the ferrum

reductum. The progressive decrease in vitamin losses, which is observed when the concentration of the acid extractant is increased, is probably a result of changes in the relative rates of solution of the vitamins and ferrum reductum.

The losses of thiamine and riboflavin during hot acid extraction from corn meal premixes containing ferrum reductum are of the same order of magnitude as from flour premixes. The relatively high ratio of limestone substrate to vitamins appears to have little effect on the vitamin losses. That the substrate may have a definite effect on the vitamin losses is shown by the experiments in which flour or starch was mixed with flour premix before hot acid extraction, with the result that vitamin losses from the premix were reduced almost to zero. Since flour is able to protect thiamine and riboflavin in the premix, it is not surprising that the relatively small amount of ferrum reductum in an analytical sample of enriched flour does not cause vitamin destruction during acid extraction.

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COMPOSITION OF THREE VARIETIES OF KANSAS-GROWN WHEAT. MINERAL ANALYSIS OF WHEAT AND SOIL[1]

W. G. SCHRENK and H. H. KING

ABSTRACT

Three different varieties of wheat, grown in 13 different locations in Kansas over a three-year period, have been analyzed for protein, ash, potassium, phosphorus, calcium, magnesium, sodium, manganese, iron, and copper. Soil samples obtained from the same locations were also analyzed for the same constituents, except for sodium. Soil pH was also measured. Rainfall data, yield, and test weight were also reported.

The ash and mineral content of wheat grown in various locations within the state varies appreciably and is correlated in general with the available nutrients in the soil. Areas producing wheat of high mineral content produced the higher protein.

The mineral content was not greatly influenced by variety. The location at which the wheat was grown was much more important than variety. Localities producing high mineral content did so consistently, indicating that differences due to rainfall and other factors during the three-year period did not significantly affect mineral content.

The increased ash content of western Kansas wheats was not due to any one element, but was the combined result of increases in each of the major constituents of the ash. A very high manganese content of wheat grain was noted from certain areas in 1943 but not the following two years. Apparently conditions were favorable for a maximum intake of manganese in these areas during the 1943 season. The minor elements in wheat and soil did not show such a definite trend across the state as the major elements.

It is well known that the mineral composition of wheat is influenced by such factors as season, locality, and variety. Excellent reviews of the literature on this subject have been given by Sullivan (8), Beeson (3), and Bailey (1). Most of these studies, however, have dealt with relatively small areas and isolated sections of the wheat-growing belt. Most of them also have not been concerned with consecutive crops grown in the same locality. In view of these facts and because of the importance of Kansas as a wheat-producing area, a study was undertaken of the mineral composition of several standard varieties of hard red winter wheat grown in various locations in the state. The results are presented in this report.

Materials and Methods

Design of the Experiment. The study was extended over a three-year growing period, with samples obtained from the same plots each

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year. In addition, soil analyses from the same plots were made for two years and average values are presented in order to correlate these data with the grain analyses. Other data presented include average rainfall, protein, ash, yield, and test weight. The data taken as a unit, therefore, combine many of the factors generally considered to have an influence on the growth of wheat, and cover the state of Kansas for wheat grown in 13 different localities, including in each location, samples of Turkey, Blackhull, and Tenmarq. The following report presents the data as obtained and the methods used for the analyses.

Analysis of Wheat Samples. Wheat samples were ground and analyzed for the following constituents: moisture, ash, protein, potassium, calcium, magnesium, phosphorus, sodium, manganese, iron, and copper. For the determination of total ash, the samples were ignited in a muffle at 600°C. for 16 hours. Protein determinations were made by the usual A.O.A.C. Kjeldahl procedure. A factor of 5.7 was used to convert per cent nitrogen to per cent protein.

Potassium was determined colorimetrically by the procedure Harris (4) recommended. Calcium was determined by titration with permanganate. After a 20-mg. sample of ash had been dissolved in the buffer solution, the method was similar to that which Wang (9) used for blood serum.

The method used for magnesium was similar to that described by Lindner (5) which makes use of titan yellow as a colorimetric reagent. This method is satisfactory for plant materials provided the pH is kept constant. It was found desirable to use 3.5 ml. of 15% potassium hydroxide rather than 1 ml. of the 40% solution as recommended by Lindner.

The remaining mineral constituents, phosphorus, sodium, manganese, iron, and copper, were determined spectrographically on a Bausch and Lomb large litrow spectrograph, using the standard solutions, spectral lines, and techniques suggested by Morris, Pascoe, and Alexander (7), with the following changes which were necessary principally because of different spectrographic equipment. Solutions of the samples were placed on electrodes as recommended by Morris *et al.* (7). The image was focused on the collimating lens and the electrode spacing was set at 1 mm. The samples were arced for 5 minutes. The excitation source was a high voltage A.C. arc at 2200 volts and 2.4 amperes. The sector was set at three-eighths open. All spectra were taken in duplicate and a set of previously prepared standards was placed on each plate. Plates were developed as recommended by Morris.

Analyses were made by use of the spectral lines recommended by Morris, and line densities were read with an A.R.L.-Dietert densitom-

eter. Per cent composition was then determined from standard curves. Cadmium was used as an internal standard.

Analysis of Soil Samples. Soil samples, obtained from the same fields which produced the grain, were analyzed for available potassium, calcium, magnesium, nitrogen, manganese, iron, copper, and phosphorus.

The soil samples were extracted with Morgan's solution (6) and the extracts analyzed for the readily available plant nutrients which were present. The procedures used for nitrogen, potassium, magnesium, phosphorus, and calcium were essentially those presented by Wolfe (10, 11) and later summarized by him (12).

The remaining constituents, manganese, iron, and copper, were determined spectrographically. The method was essentially the same as with plant material. However, a few changes were required due to the presence of the salts used in Morgan's extracting solution. Standards were prepared in the presence of the extracting solution for comparison purposes, because of the effect of large quantities of salts on the line intensities of the minor elements. Spectrographic plates were taken under the conditions previously described and concentrations calculated in the same manner. As sodium was present in the extracting solution it was not determined in the soil.

Results and Discussion

An outline map of Kansas showing the location of the test plots under the supervision of the agronomy department is shown in Fig. 1. The samples represent widely scattered localities and quite different growing conditions. The wheat samples were secured from the same plots all three years. During the 1944 season no samples were available from Tribune or Smith Center.

Table I presents data of the United States Weather Bureau for the average rainfall at each location. The variations in rainfall, in conjunction with differences in basic soil types, could be responsible for quite different results at different locations. Rainfall at the various locations was average or slightly higher during the period of the survey.

Table I also gives the average yield and test weight at each location. Good yields were obtained in most instances. In 1943, however, yields at Meade and Dodge City averaged only 5.0 and 7.4 bushels per acre respectively. Hays produced 9.5 and Belleville 8.1 the same year. These locations produced the lower average yields for the three-year period as shown in the table. Average yields for all plots sampled were respectively 20.6, 30.0, and 33.4 bushels per acre during the three-year period with an overall average of 28.0 bushels

per acre for the survey. Test weight values varied somewhat with location, with the Tribune, Kingman, and Wichita fields showing the highest averages. The Blackhull variety exhibited a slightly higher test weight than the two other varieties studied.

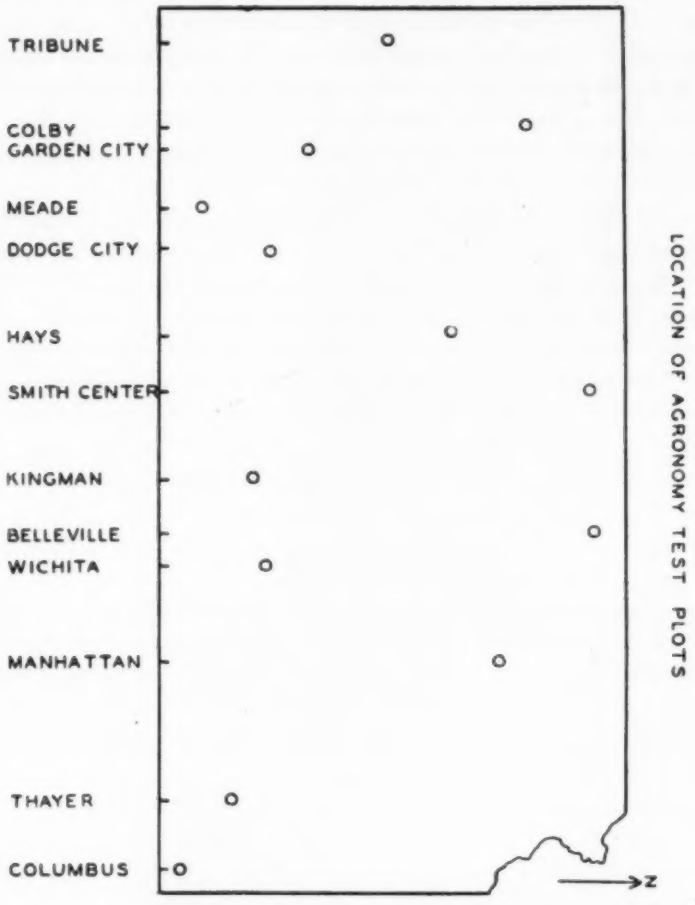

Fig. 1. Outline map of Kansas showing locations from which wheat samples were obtained.

Ash and Protein Content. Table II presents data regarding average ash and protein content of the wheat samples. The variation in total ash, or mineral content, at the various locations is striking; in fact, location was more important than other conditions in determining ash content. High ash samples usually occurred at the same location each year. The highest values for mineral content were obtained from the southwestern and central areas of the state, i.e., at Meade and Dodge City and at Hays. Furthermore, these areas were

TABLE I

MEAN RAINFALL, YIELD, AND TEST WEIGHT OF WHEAT GROWN AT VARIOUS LOCATIONS IN KANSAS
(Averages for 1943–1945 inclusive)

Location	Average annual rainfall	Yield per acre	Test weight
	Inches	*Bu.*	*lb./bu.*
Tribune	15.8	40.9	60.4
Colby	17.7	48.9	59.4
Garden City	18.5	29.1	57.9
Meade	20.4	22.4	56.2
Dodge City	19.8	21.6	56.9
Hays	22.6	18.1	56.4
Smith Center	21.9	27.7	58.4
Kingman	29.5	22.8	61.1
Belleville	26.2	19.3	53.5
Wichita	29.7	28.1	60.3
Manhattan	31.0	26.9	58.3
Thayer	39.9	32.2	57.9
Columbus	41.8	31.2	57.0

consistently high all three years. There were no consistent varietal differences apparent, location being the more important factor.

The protein content of the wheat varied in a somewhat similar manner, although a general trend from one year to the next was observed. In 1943 protein content was highest, with somewhat lower levels in 1944 and 1945. Areas which produced high mineral content also produced wheat of high protein content; the top five locations with regard to ash content included the top four locations in protein.

TABLE II

MEAN ASH AND PROTEIN CONTENT OF WHEAT GROWN AT DIFFERENT LOCATIONS [1]
(Mean values for 1943–1945 inclusive)

Location	Ash	Protein
	%	%
Tribune	1.86	14.8
Colby	1.92	15.0
Garden City	2.09	16.9
Meade	2.43	17.2
Dodge City	2.36	16.3
Hays	2.33	17.7
Smith Center	1.93	16.7
Kingman	1.82	13.5
Belleville	2.33	13.0
Wichita	1.91	14.0
Manhattan	2.03	13.6
Thayer	1.91	12.4
Columbus	2.00	11.7

[1] All data on this and the following tables are on a moisture-free basis.

The fifth location, which consistently produced wheat of high mineral content but of lower protein, was the Belleville field. As would be expected, the areas which received the most rainfall produced good yields of slightly below average ash content and low protein. Kingman, with the lowest ash content, also produced wheat of lower protein content.

Major Mineral Elements. Table III contains data on the four metallic elements found in greatest amount in wheat—potassium, phosphorus, magnesium, and calcium. The oxides of these four elements account for well over 90% of the total ash. Wheat samples obtained from western fields averaged higher in mineral content in all cases, presumably due to the higher levels of available nutrients in the soil.

TABLE III

MEAN POTASSIUM, PHOSPHORUS, MAGNESIUM, AND CALCIUM CONTENT IN THREE VARIETIES OF KANSAS WHEAT GROWN AT VARIOUS LOCATIONS
(Means for 1943–1945 inclusive)

Location	K	P	Mg	Ca
	%	%	%	%
Tribune	0.44	0.34	0.133	0.051
Colby	0.41	0.36	0.139	0.048
Garden City	0.40	0.42	0.152	0.051
Meade	0.43	0.53	0.157	0.049
Dodge City	0.45	0.50	0.153	0.057
Hays	0.45	0.41	0.142	0.066
Smith Center	0.40	0.38	0.122	0.045
Kingman	0.37	0.34	0.104	0.036
Belleville	0.42	0.50	0.136	0.047
Wichita	0.38	0.39	0.109	0.038
Manhattan	0.36	0.44	0.131	0.035
Thayer	0.35	0.47	0.116	0.041
Columbus	0.36	0.53	0.129	0.036

Potassium averaged highest in the samples obtained from the Dodge City, Meade, and Hays fields. These fields are also among those high in available potassium, as shown in the soil analyses presented in Table V. Likewise, the lowest potassium levels in the grain were from fields of lowest potassium content in the soil.

Phosphorus data show an interesting deviation from the correlation with the soil data. The samples obtained from the Columbus and Thayer fields were taken from plots which had been fertilized with additional phosphate. The general downward trend in phosphorus as the eastern border of the state was approached was reversed at these locations, due undoubtedly to the added fertilizer these plots received. Unfortunately, samples of wheat grown on adjacent untreated plots were not available for comparison. Samples from

the Belleville field in north central Kansas also contained larger amounts of phosphorus than other fields of about the same level of available phosphorus in the soil.

Magnesium in the grain also averaged higher in the western samples. The lowest magnesium content in grain was found in samples from Kingman, which also had the lowest magnesium content in the soil. The general trend in magnesium is not quite so evident as were those of potassium and phosphorus.

Calcium also follows the generalization that western Kansas grains are higher in the inorganic constituents of the ash, although the highest average calcium in the grain was not produced on the test plots of highest calcium. The Hays test plots produced wheat of the highest calcium content every year. Wheat produced at Hays had the highest average protein content. Other western Kansas test plots also produced wheat of higher calcium content than those located in eastern Kansas.

The higher mineral content of the western Kansas wheats is due not to any one element, but rather to the combined effect of the several major constituents of the ash. The variation in mineral content of the grain is much less than the variation of the corresponding element in the soil. For example, there was an eightfold variation in available calcium in the soil and only a twofold variation in the calcium content of the grain.

Minor Mineral Elements. Table IV contains data regarding the minor elements (manganese, iron, copper, and sodium) included in the study. Although a number of other elements have been shown by Sullivan (8) to be constituents of wheat, they have not, as yet, been quantitatively determined in this laboratory.

These minor elements vary more than the major constituents of the ash. In several cases the average figures in Table IV do not give an indication of yearly variability. In 1943 wheat grown at Meade and Dodge City contained about four times as much manganese as that grown farther east. This trend was not particularly noticeable the following two years. Since it is known that the availability of manganese is influenced greatly by soil moisture, it may be assumed that more optimal conditions for absorption of manganese occurred in this area in 1943 during the proper stage in plant growth to produce a grain high in manganese; iron, also, showed a somewhat similar trend, being higher in the western area in 1943 than in the two following years.

Sodium content of the grain was quite uniform throughout the state all three years. It was slightly lower in 1945 than the previous two years. In general, the sodium content of Kansas wheat was some-

what higher than values reported by Morris *et al.* (7) and Sullivan (8) on their samples.

Copper is known to be essential in plant growth. Barham *et al.* (2) have recently suggested its possible correlation with the starch content of the sorghum grains. This study seems to indicate that the areas included in this survey contain sufficient copper for proper plant growth. The copper content of the wheat, although varying somewhat, was generally uniform throughout the state.

TABLE IV

MEAN SODIUM, IRON, MANGANESE, AND COPPER CONTENT IN THREE VARIETIES OF KANSAS WHEAT GROWN AT VARIOUS LOCATIONS
(Crop years 1943–1945 inclusive)

Location	Na	Fe × 10^3	Mn × 10^3	Cu × 10^3
	%	%	%	%
Tribune	0.016	0.66	0.55	0.69
Colby	0.015	0.79	0.57	0.55
Garden City	0.020	0.85	0.70	0.65
Meade	0.022	1.21	0.81	0.77
Dodge City	0.020	1.00	0.55	0.74
Hays	0.019	0.70	0.38	0.65
Smith Center	0.014	0.61	0.37	0.43
Kingman	0.014	0.50	0.26	0.53
Belleville	0.018	0.70	0.49	0.71
Wichita	0.014	0.53	0.48	0.67
Manhattan	0.015	0.67	0.29	0.55
Thayer	0.016	0.65	0.42	0.64
Columbus	0.017	0.70	0.43	0.70

Available Nutrients in Soil. A definite correlation exists between available nutrients (Table V and VI) and the wheat analyses. The soil samples were obtained in 1943 and 1944 from the same plots on which the wheat was grown. In general, the trend in readily available nutrients follows an order dependent on rainfall. Those areas which have received more rainfall are those in which nutrient materials have been leached out of the soil.[2]

Soil data indicate that the major nutrients are still in good supply in Kansas except in the extreme southeast. This area receives the greatest rainfall and has been under cultivation longer than other areas of the state. This section of the state produces wheat of lower protein content, but it is not a part of the main wheat-producing belt of the state. The soils of the extreme southeastern part of the state show favorable response to the additions of commercial fertilizers.

The data presented in Table V show that there is a decided variation in the readily available potassium, calcium, phosphorus, and nitro-

[2] The general classification of the soil in each location may be readily obtained from the map of Kansas entitled "Natural Agricultural Resource Areas of Kansas" compiled for Region 5, Soil Conservation Service, by C. L. Fly, soil scientist.

TABLE V

MEAN AVAILABLE POTASSIUM, CALCIUM, PHOSPHORUS, AND NITROGEN CONTENT, AND pH IN KANSAS SOIL [1] FROM VARIOUS LOCATIONS
(Crop years 1943–1944 inclusive)

Location	pH	Parts per million			
		K	Ca	P	N
Tribune	6.70	222	9400	51.7	100
Colby	6.58	265	4580	49.8	115
Garden City	7.04	146	8600	47.1	98
Meade	6.65	253	3370	40.3	117
Dodge City	6.28	204	4060	42.1	109
Hays	6.59	191	3300	48.8	71
Smith Center	6.17	181	3040	37.8	77
Kingman	5.95	78	1960	26.0	86
Belleville	6.18	118	2830	27.4	100
Wichita	6.23	148	2730	30.2	124
Manhattan	6.61	114	2980	23.7	91
Thayer	6.08	25	1940	18.1	64
Columbus	6.00	23	1140	14.8	67

[1] 0–20 inches.

gen content of the soils as well as in the pH of the soil solutions in the various localities. For example, potassium varies from over 250 ppm. in the west to 23 ppm. in the southeast. Calcium shows similar variations. pH measurements also indicate variations in the presence of the base-producing ions, with a range in pH values of from 7.04 to 5.95. The areas of high nutrient availability coincide with those producing the wheat of highest ash and protein content.

TABLE VI

MEAN AVAILABLE MAGNESIUM, MANGANESE, IRON, AND COPPER IN KANSAS SOIL [1] FROM VARIOUS LOCATIONS
(Years 1943–1944 inclusive)

Location	Parts per million			
	Mg	Mn	Fe	Cu
Tribune	172	35	36	3.0
Colby	144	43	31	3.1
Garden City	190	25	33	3.2
Meade	154	69	35	3.3
Dodge City	150	61	45	3.3
Hays	262	38	27	2.7
Smith Center	252	40	31	3.1
Kingman	59	47	32	2.4
Belleville	110	45	26	3.1
Wichita	148	49	41	3.2
Manhattan	214	62	29	3.5
Thayer	117	65	41	3.7
Columbus	123	42	40	3.4

[1] 0–20 inches.

The data on soil samples from the Kingman field offer an opportunity for an interesting evaluation of soil and grain analyses. Kingman is relatively low in all the nutrients for which analyses were made, although not the lowest in every case. The grain from this area consistently had the lowest ash content, and protein was also quite low. This would indicate an effect due to the decreased availability of the required elements even though they may be present in amounts which may be above the minimum generally recognized as required for the crops. Interestingly enough, the wheat grown at the Kingman field had the highest test weight during the period under study. The decreased availability of the necessary elements is also noticed in Table VI. For example, magnesium is very low in comparison to other fields. The other minor elements which were included in the survey were also well below average. The lowered availability of the elements in this area is reflected in the generally decreased quantity of each of the elements in the grain as indicated in Tables III and IV.

The data presented in Table VI, which include magnesium, manganese, iron, and copper, do not show entirely the same general and definite trend as was the case with the elements included in Table V. The copper content of the soil is fairly uniform in the fields sampled, although in the central area it is somewhat lower than in the others. Magnesium is higher in the west, and as noted previously is lowest in the samples taken from the Kingman field. Manganese varies considerably in different localities; although the manganese was very high in samples of grain from the Meade and Dodge City fields in 1943, several other fields were almost as high in the manganese content of the soil. Iron varied somewhat over the state, also, but the trend was not regular. The central area was lower in available iron, and the samples from Thayer and Columbus contained more iron, in general, than did those from other areas.

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AMYLOSE CONTENT OF INDIAN CORN STARCHES FROM NORTH, CENTRAL, AND SOUTH AMERICAN CORNS [1]

ROY L. WHISTLER [2] and PAUL WEATHERWAX [3]

ABSTRACT

Analyses of starch samples from 39 different Indian corns indicate iodine adsorption values of 44.4–56.6 mg. per gram of starch or amylose contents of 22.2–28.3%, the average being 24–25%. Thus, these starches have usual amylose contents.

Corn starches which have previously been analyzed for amylose content may be classified into two principal groups: (A) those from waxy or low amylose corn varieties which contain 0–6% amylose, and (B) those from practically all other corn varieties, including both open-pollinated and hybrid varieties of dents, pop, and sweet corns which contain 25–29% amylose. Only a few waxy or low amylose starches are known. Recently a sugary mutant (3) has been found to contain 50–65% amylose and hence cannot be classified in either group. All other corn starches on which published data are available fall into the second group.

Recently there have become available a number of corn varieties from widely different localities ranging from Arizona and New Mexico to southern South America. For the most part, these samples represent relatively unimproved varieties which have been grown by the Indians for centuries. They include a variety of different pigmenta-

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tions and kernel sizes and shapes. Endosperm types range from horny to mixtures of horny and floury and to almost entirely floury. It was of interest, therefore, to analyze the starches from a number of these samples to determine whether any of them might contain unusual amounts of amylose.

Materials and Methods

Corn Samples. Samples of the different varieties of corn were collected by one of the authors (Weatherwax) during the course of a survey which extended from New Mexico and Arizona to South America. Funds for the survey were made available through the generosity of the Guggenheim Foundation, Indiana University, and the Funk Brothers Seed Company. Sources of the various samples are shown in Table I. Sample ears were obtained from the native farms or fields or from native markets.

Preparation of Starch. Four or five representative kernels from each ear were selected, the grain soaked in water for 2 or 3 hours and the pericarp of each removed with a knife. The aleurone was also scraped away as completely as possible. After cutting out the germ, the remainder of the kernel was ground to pass a 60-mesh screen by a laboratory Wiley mill. In one instance, however, in order to make a comparison of the horny and floury endosperms, the two were separated by first grinding out the floury part by means of a dental drill. The residual horny portion was then ground in a Wiley mill. Samples of powdered endosperm varied in weight from 0.2 to 1.5 g.

Fat was removed from the powdered endosperm by four successive one-hour extractions with refluxing 85% methanol (6, 7). Approximately 10 ml. of methanol was used for each reflux period, and the mixture was stirred briefly at frequent intervals. After each reflux period, the sample was filtered on a microfunnel, washed well with methanol, and then mixed with 10 ml. of fresh methanol. After the final extraction, the crude starch was filtered, washed with methanol, and air-dried.

Analytical Methods. Since the quantity of material in each sample was small, moisture determinations were made by the following indirect procedure: A number of samples were spread out in thin layers and allowed to equilibrate with the atmosphere along with a 6–8 g. sample of commercial corn starch previously methanol-extracted in the same manner as the small samples. At the end of four days, triplicate moisture determinations were made on the large sample and its moisture content was assumed to represent that of the small samples.

Amylose was determined by a method only slightly different from

that described by Bates, French, and Rundle (1) and modified by Wilson, Schoch, and Hudson (8). Starch samples were dissolved in 0.5 *N* potassium hydroxide by allowing the mixture to stand for approximately 24–48 hours at 0°C. in an atmosphere of nitrogen. After the solution of the starch, a small amount of undissolved protein and possibly some cellulosic fiber remained in the solution. Since this material, which constituted less than 1% of the sample, was found not to affect the result of the subsequent potentiometric iodine determination, it was allowed to remain in the solution. Iodine values were corrected for free iodine and hence represent the total number of milligrams of iodine bound per gram of starch.

Sources of Error. The method of indirect moisture determination leads to little, if any, error. This was shown in several instances by determining moisture on the micro samples as well as on the large samples. In all the tests made, moisture values of the small samples were identical with those of the large samples.

Both the protein and ash in the endosperm serve as inactive diluents in the starch. The ash content in the endosperm of several varieties of commercial corn has been shown (4) to vary from 0.22 to 0.46%. Similar ash contents were assumed for the endosperms examined in this investigation. These values were below the limits of error for the survey method used here, and hence the ash content of the endosperm is disregarded in the subsequent calculations of amylose content.

The greatest error encountered is that involved in the estimation of protein. In an examination of the protein content of the endosperm from 11 varieties of commercial corn, Earle, Curtis, and Hubbard (4) found a variation of 6.7 to 12.8% with an average of 9.4%. Hansen, Brimhall, and Sprague (5), in examining a wide variety of endosperm types, noted protein contents which varied about 5 to about 22% with an average of approximately 12%. The results show that roughly half of the protein is extractable with alcohol. This soluble protein would be removed during the defatting treatment described above. Consequently, it was assumed for the purpose of calculating amylose contents that the methanol-extracted starch herein investigated contained approximately 5% protein. Potentiometric iodine values were correspondingly corrected. These assumptions would indicate an analytical error of about $\pm$ 5%.

A test of this procedure was obtained by applying it to the analysis of a sample of corn whose starch is known to have an iodine sorbing value of 51.7 mg. per gram. A value of 51.0 was obtained for the starch by this procedure. Amylose content in per cent may be calculated by assuming that pure amylose sorbs 200 mg. of iodine per gram (unpublished data of I. A. Wolff and R. L. Whistler).

In several instances where sufficient grain was available, the starch was isolated and purified by the method of Brimhall, Sprague, and Sass (2). Analytical values of the starch indicated the same amylose contents as found with the microtechnique described above.

Results and Discussion

Analyses of starch samples from 39 different Indian corns indicate iodine sorption values (Table I) of 44.4 to 56.6 mg. per gram of starch

TABLE I

AMYLOSE CONTENT OF INDIAN CORNS

Sources	Iodine affinity
	mg./g.
San Juan, Pueblo, New Mexico, 1819.12 [1]	50.6
Retalhuleu, Guatemala, 1849.4	52.6
San Juan, New Mexico, 1819.26	52.0
San Juan, New Mexico, 1819.16	46.8
Huancayo, Peru, 1872.66	45.8
Huancayo, Peru, 1872.103	51.4
Huancayo, Peru, 1872.134	51.6
Navajo, Ft. Defiance, Arizona, 1877.19	48.2
Navajo, Ganado, Arizona, 1875.12	51.0
Navajo, Ganado, Arizona, 1875.17	48.4
Arequipa, Peru, 1860.4	50.8
Arequipa, Peru, 1860.6	48.0
Chimaltenango, Guatemala, 1842.3	45.6
Antigua, Guatemala, 1846.1	48.6
Calca, Peru, 1864.3	50.2
Chacan, Peru, 1865.17	46.2
Hopi, Moenkopi, Arizona, 1831.10	51.0
Huancayo, Peru, 1872.82a	47.2
Jemez, Pueblo, New Mexico, 1823.3	49.6
Quetzaltenango, Guatemala, 1845.5	45.6
Quito, Ecuador, 1857.8	48.6
Paguate Pueblo, New Mexico, 1824.9	50.4
Paguate Pueblo, New Mexico, 1824.13	48.4
San Idelfonso Pueblo, New Mexico, 1818.3	49.6
Calca, Peru, 1864.15	47.2
Santa Ana Pueblo, New Mexico, 1822.5	50.2
Santa Clara Pueblo, New Mexico, 1820.5	55.0
Santa Clara Pueblo, New Mexico, 1920.15	51.8
Santa Clara Pueblo, New Mexico, 1920.21	56.6
Santo Domingo, Ecuador, 1859.2	53.4
Santo Domingo, Ecuador, 1859.10	48.8
Tecpan, Guatemala, 1843.3	46.6
Tesuque Pueblo, New Mexico, 1817.2	54.2
Tesuque Pueblo, New Mexico, 1817.3	48.2
Tesuque Pueblo, New Mexico, 1817.5	45.0
Toluca, Mexico, 1834.16	49.4
Toluca, Mexico, 1834.17	44.4
Toluca, Mexico, soft white, 1834.18	51.2
Zuñi Pueblo, New Mexico, 1829.3	47.0
Indiana Hybrid 644, floury endosperm	49.6
Indiana Hybrid 644, horny endosperm	48.6

[1] Numbers are those identifying the sample in the collection of Paul Weatherwax.

or amylose contents of 22.2 to 28.3%, the average being 24–25%. This compares with an amylose content of 26% (iodine sorption value of 51.7) for seven starches from standard corn belt corns.

This uniformity of composition occurs despite the fact that the corn samples were obtained from widely separated areas and differed considerably in their appearance, coloration, and relative amounts of floury to horny endosperm.

Although the analytical values obtained in this survey may be in slight error, they clearly do not indicate the presence of waxy starches or starches with exceptionally high amylose contents.

Analyses of the separated horny and floury endosperms of a standard corn belt corn (Indiana Hybrid 644) indicate about the same amylose content for both types of starch.

Acknowledgment

The authors wish to express their appreciation to Mrs. Mary Hammel and Mrs. Iva Buschman for their assistance in the analytical determinations.

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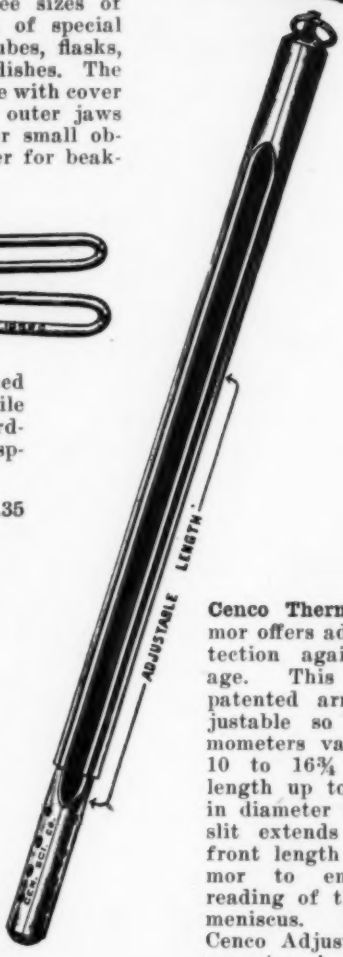

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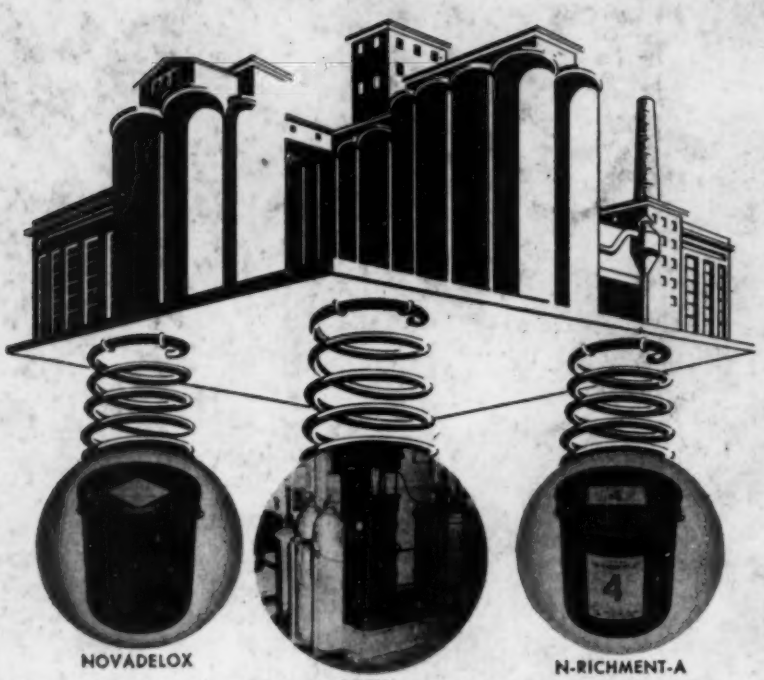

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